RELATIONSHIP BETWEEN *EPSPS* COPY NUMBER, EXPRESSION, AND LEVEL OF RESISTANCE TO GLYPHOSATE IN COMMON WATERHEMP (*AMARANTHUS RUDIS*) FROM KANSAS

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Abstract

Common waterhemp (*Amaranthus rudis*) is a problematic weed species of cropping systems throughout the Midwestern states, including Kansas. Recently, waterhemp populations from Kansas were found to have evolved resistance to the widely used herbicide glyphosate as a result of amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS)*, the enzyme target of glyphosate. The objectives of this research were to 1) perform glyphosate dose-response study and determine the relationship between relative *EPSPS* genomic copies and *EPSPS* gene expression in glyphosate-resistant waterhemp, and 2) characterize the genomic configuration and distribution of *EPSPS* copies using florescence in situ hybridization (FISH) in three glyphosate-resistant waterhemp populations. Waterhemp populations from eastern Kansas were screened with 868 g ae ha^{-1} (field used rate) of glyphosate, and genomic DNA and total RNA was isolated from the survivors to determine the *EPSPS* genomic copies and *EPSPS* gene expression relative to the *acetolactate synthase* (*ALS*) gene using qPCR. Furthermore, waterhemp specific *EPSPS* probes were synthesized to perform florescence in situ hybridization (FISH) on these glyphosate-resistant plants. Results of these experiments indicate a positive correlation between level of glyphosate resistance, *EPSPS* copies, and their expression. As expected, a negative correlation was found between shikimate accumulation and *EPSPS* copies. Sequencing of the *EPSPS* gene showed no presence of the proline 106 mutation, which is known to be associated with glyphosate resistance suggesting that an insensitive *EPSPS* enzyme was not involved in the mechanism of glyphosate resistance. FISH analysis of resistant plants illustrated presence of amplified *EPSPS* copies on two homologous chromosomes, likely near the centromeric region. . This is the first report demonstrating a positive relationship between *EPSPS*

copies and expressions, as well as chromosome configuration of *EPSPS* copies in glyphosate-resistant waterhemp from Kansas.

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Chapter 1 - Literature Review

Glyphosate

History of Glyphosate

The most extensively used herbicide, glyphosate was originally synthesized in 1950 by the Swiss chemist Dr. Henri Martin while working at the small pharmaceutical company Cilag (Dill *et al.* 2010; Duke and Powles 2008). This company was purchased by Johnson and Johnson, which sold off Cilag's research samples, with the sample of glyphosate eventually tested by Monsanto Inc. (Inorganic Division), initially as a water softening agent, and then as a herbicide. When its herbicidal properties were found too low, it was given to Dr. John E. Franz and his team, at Monsanto Inc. who synthesized a more potent form of chemical, which was commercialized by Monsanto Company under the product name Roundup (Alibahai and Stallings 2001; Dill *et al.* 2010, Duke and Powles 2008). Glyphosate has many unique properties, as described in the next section, which make this herbicide an exceptional product.

Glyphosate: Mode of Action

Glyphosate (*N*-(phosphonomethyl)glycine) (Figure 1.1), a derivative of the amino acid glycine, was known to be an effective herbicide since it was released commercially, however, the exact chain of events causing plant mortality was not originally understood (Dill *et al.* 2010). Early work by Steinrucken and Amrhein (1980) to elucidate its mode-of-action of this herbicide, found that it caused an accumulation of shikimate in plant tissues, due to the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, impairing conversion of shikimate to chorismate in the shikimate pathway (Figure 1.2) (Amrhein *et al.* 1980; Duke and Powles

2008; Healy-Fried *et al.* 2007; Schonbrunn *et al.* 2001). This metabolic pathway facilitates the synthesis of aromatic amino acids: phenylalanine, tyrosine, and tryptophan; importantly, this pathway is only present in plants, bacteria, and fungi, and is not in animals (Alibahai and Stallings 2001; Herrmann and Weaver 1999). Herrmann and Weaver (1999) further elucidated the steps involved in the shikimate pathways, starting with phosphoenolpyruvate (PEP) and erythrose-4-phosphate. This is an extremely important pathway, as up to 35% of the dry weight of a plant is made up of the small molecules synthesized here (Alibahai and Stallings 2011). EPSPS catalyzes the conversion of shikimate-3-phosphate to 5-enolpyruvylshikimate-3-phosphate (Alibabhai and Stallings 2001; Schonbrunn *et al.* 2001). Duke and Powles (2008) suggest that glyphosate, an analogue of PEP, acts as a competitive inhibitor of EPSPS, leading to the observed accumulation of shikimate. Plant mortality is caused by either carbon drain from other important pathways into the shikimate pathway, or due to lack of aromatic amino acid biosynthesis (Duke and Powles 2008).

Glyphosate is very unique, selectively targeting EPSPS with high specificity to the EPSPS enzyme (Duke and Powles 2008). Glyphosate is readily absorbed into plant tissues, where it translocates via phloem, to sink tissues, most importantly the meristem where EPSPS is highly expressed, as well as roots and storage organs (Dill *et al.* 2010; Duke and Powles 2008). The cellular changes associated with glyphosate treatment, besides shikimate accumulation, include reduced photosynthesis, a decline in chlorophyll levels, and an increase in carotenoid pigments (Baylis 2000). Whole plant symptoms take longer to develop, and include chlorosis, plant stunting, and reduced apical dominance. Glyphosate is slightly more effective in controlling grass species than broadleaf plants on average, and is ideal in targeting perennial

plants, due to its systemic nature of translocation , though higher doses are necessary (Baylis 2000).

This herbicide also has excellent environmental qualities as well. Duke and Powles (2008) explain that it is one of the least toxic herbicides to animals, and that it is even less acutely toxic than aspirin or sodium chloride. It is not known to affect reproductive tissues. Baylis (2000) states that glyphosate does not bio-accumulate in animal tissues. Glyphosate binds tightly to soil colloids, inactivating the herbicide and preventing it from leaching into groundwater (Baylis 2000, Duke and Powles 2008). This binding can be reversed by bacteria which metabolize the chemical, contributing to the relatively short environmental half-life. This degradation is rapid, and occurs in both soil and water, leaving behind only one major metabolite, aminomethylphosphonic acid (AMPA), which is also quickly degraded (Rueppel *et al.* 1977). The chemical is stable in sunlight, and does not have any negative effects on soil fauna and microflora, as well as on honeybees (Baylis 2000, Rueppel *et al.* 1977). One issue with glyphosate is that calcium and magnesium can act as antagonists in hard water and deactivate it, however, addition of ammonium methyl sulfate (AMS) prior to application prevents this. Fernandez-Cornejo *et al.* (2014) argue that glyphosate is typically much more environmentally friendly than other herbicides. The above beneficial characteristics of glyphosate combined with development of Roundup Ready crop technology, facilitated extensive use of glyphosate and thus, this herbicide has substantial impact on agriculture around the globe.

Agricultural Impact of Glyphosate

Glyphosate has become the most popular herbicide worldwide because of its inherent safety and effective, non-selective weed control (Alibahai and Stallings 2001; Healy-Friend *et al.* 2007; Pline-Srnic 2006). According to Baylis (2000) and Woodburn (2000) it is the world's

largest selling and fastest growing agrochemical. Fernandez-Cornejo *et al.* (2014) states that glyphosate has been the most popular and extensively used herbicide in the United States of America since the year 2001, and use of this herbicides is steadily increasing since then. Duke and Powles (2008), and Woodburn (2000) state one of the major reasons for this increase in popularity is the expiration of Monsanto Company's patent on the chemical in 2000. Glyphosate is a globally used chemical, and has been registered for use in 130 countries, with an estimated global volumes of 600 kilotons a year (Dill *et al.* 2010). About 100 annual grass and broadleaf weed species, as well as 60 perennial weed species are known to be controlled by glyphosate, which has increased in recent years.

The creation of transgenic crops that can withstand glyphosate application, coupled with implementation of conservation tillage practices in agriculture, have further increased the usefulness and popularity of glyphosate (Baylis 2000; Dill *et al.* 2010, Duke and Powles 2008; Woodburn 2000). While glyphosate was originally utilized to control weeds in ditches, right-of-ways, and fallow agricultural fields as well as other non-crop areas, the advent of minimum tillage gave it increased usefulness as a preplant herbicide, and with the release of glyphosate-tolerant (GT) crop technology, it has become a season-long chemical option for weed control. Adoption of minimum tillage systems has resulted in reduced fuel use, labor costs, and soil erosion. Conservation or minimum tillage practices also improve soil structure and organic matter content, as well as water permeation and carbon sequestration, highly beneficial to the environment (Dill 2005, Duke and Powles 2008). This combination of conservation tillage and herbicide-tolerant crop technology used in tandem is extremely popular, with 33% of all corn planted in 2005, 86% of all soybeans in 2006, and 32% of all cotton in 2007 to no-till production. Compare this with only 19% of corn, 36% soybeans, and 32% cotton planted in no-

till being conventional, non-genetically modified crops grown in concert with conservation tillage practices (Fernandez-Cornejo *et al.* 2014). However, due to this excessive utilization of glyphosate, and only glyphosate, in many production systems, there has been a global shift towards the evolution of glyphosate resistance in weed species, as well as (GT) crops becoming volunteer weeds themselves (Duke and Powles 2008; Owen and Zelaya 2005).

Glyphosate-Tolerant Transgenic Crops

The popularity of GT transgenic crops, and as such, glyphosate, relied on them being economically and environmentally favorable, as well as highly effective, simplifying weed control in many agricultural systems (Dill 2005; Duke and Powles 2009; Fernandez-Cornejo *et al.* 2014). GT soybeans were first made commercially available in 1996 by Monsanto, and adoption of GT crops, and transgenic crops in general, has been extremely rapid since (Brookes and Barfoot 2013; Dill *et al.* 2008). GT crops currently include soybeans (*Glycine max*), corn (*Zea mays*), cotton (*Gossypium hirsutum*), canola (*Brassica napus*), sugarbeet (*Beta vulgaris*), and alfalfa (*Medicago sativa*) (Duke and Powles 2008; Duke and Powles 2009). Brookes and Barfoot (2013) reported that this adoption has been extremely beneficial worldwide, with direct farm income from soybeans, corn, cotton, and canola equaling $19.8 billion in 2001, totaling $98.2 billion since genetically modified (GM) crops were introduced in 1996. Global production of corn and soybeans increased by 195 million and 100 million tonnes, respectively, since GM seed reached the market. Farmers in developing countries reaped 51.2% of this economic gain. Dill *et al.* (2010) states that the United States, Canada, Argentina, and Brazil grew the most GT crops worldwide in 2008. Five GT crops were being grown on 74 million hectares, with 54.2 million of soybeans, 13.2 million of corn, 5.1 million cotton, and 2.3 million of canola in 13 different countries. As of 2006, 90% of soybeans, 70% of cotton, and 75% of canola grown in

the United States and Canada had glyphosate tolerance traits, with nearly 100% of soybeans being GT in Argentina (Duke and Powles 2008). Duke and Powles (2009) also state that more than 80% of 120 transgenic crops grown annually contain the glyphosate tolerance trait. Corn, cotton, and soybean are the most commonly grown GM crops grown in the United States, being planted to 169 million acres in 2013, half of the entire land area devoted to crop production in the country (Fernandez-Cornejo *et al.* 2014). Also, 93% of soybean, 85% of corn, and 83% of cotton grown in the United States also has transgenic tolerance to glyphosate.

Early development of GT technology utilized cultured plant cells and tissues exposed to increasingly higher doses of glyphosate, with resistance developing due to increased EPSPS expression, gene amplification, and altered enzyme stability (Bradshaw *et al.* 1997; Pline-Srnic 2006). However, these adaptations were not heritable, and therefore, the research was transitioned to mutagenesis and genetic transformation. The methods that were utilized in creating GM plants include site-directed mutagenesis of plant DNA. Glyphosate tolerance by metabolism is facilitated by insertion of two different genes, *GOX* and *gat*, encode for enzymes that metabolize glyphosate, result in a low level of tolerance. Unfortunately, the level of tolerance afforded by these inserted genes was not commercially acceptable. The most successful transgenic approach to production of GT crops was achieved by insertion of the *CP4* gene from *Agrobacterium* encoding a glyphosate-insensitive EPSPS enzyme (Duke and Powles 2008; Pline-Srnic 2006). The entire *CP4* gene, including the promoter region, was inserted into soybean and canola to create highly GT plants, with canola also receiving the *GOX* gene for glyphosate degradation. These genes increased glyphosate tolerance approximately 50-fold relative to a susceptible plant. Transgenic corn was created by modifying its *EPSPS* gene to produce a glyphosate insensitive enzyme (Duke and Powles 2008).

There has been some concern with transgenic glyphosate tolerance gene flow between crops and wild and weedy relatives, such as between canola and wild mustard (*Brassica rapa*) in Canada, corn and teosinte (*Euchlaena mexicana*) in Mexico, and weedy relatives of cotton in Central and South America. However, the only major occurrence of this was between canola and wild mustard, with little evidence of this in other areas. The main unintentional transgene flow events occurred between GT crops into organic and conventional crops.

Glyphosate Resistance in Weed Species

The evolution of glyphosate resistance in weeds was long thought to be unlikely due to the existence of few naturally-resistant plants, despite decades of use, most likely due to the chemical's unique properties, as reported by Bradshaw *et al.* (1997). Examples cited include the low soil residual activity of glyphosate, the specificity and effectiveness of glyphosate, as well all examples of glyphosate resistance being induced through genetic engineering. However, the first glyphosate-resistant (GR) weed, rigid ryegrass (*Lolium rigidum*) was discovered in Australia in 1996, the same year GT crop technology was first released in the market (Baerson *et al.* 2002). As of February 2015, there are currently 32 glyphosate resistance weeds in 22 different countries around the world (Heap 2015). The main factor driving this increase in resistance to glyphosate is overreliance on this single chemical option for weed control in GT minimum till production in many farmers' cropping systems (Duke and Powles 2009; Fernandez-Cornejo *et al.* 2014; Powles 2008; Preston and Wakelin 2008; Shaner *et al.* 2012). The popularity of glyphosate and GT crops will continue this trend unless other weed control strategies and herbicide modes of action are utilized. Producers have had little economic reason to diversify their weed control arsenal until recently with the rapid increase in GR weeds, due to the overreliance on glyphosate (Fernadez-Cornejo *et al.* 2014; Powles 2008). Examples of weed species that evolved resistance

to glyphosate include hairy fleabane (*Conyza bonariensis*) (Urbano *et al.*2007), horseweed (*Conyza canadensis*) (Koger *et al.* 2004; Main *et al.* 2004; Mueller *et al.* 2003; VanGessel *et al.* 2001; Zelaya *et al.* 2004), Italian ryegrass (*Lolium perenne* spp. *multiflorum*) (Dickson *et al.* 2011, Perez *et al.* 2004, Perez-Jones *et al.* 2005; Perez and Kogan 2003), Palmer amaranth (*Amaranthus palmeri*) (Culpepper *et al.* 2006; Norsworhy *et al.* 2008; Steckel *et al.* 2008; Teaster and Hoagland 2013), and rigid ryegrass (Feng *et al.* 1999; Lorraine-Colwill *et al.* 2001; Pedersen *et al.* 2007; Powles *et al.* 1998; Prately *et al.* 1999; Simarmata *et al.* 2003,2005). These weeds all have resistance to glyphosate in novel ways (described below), and more weed species may develop resistance as long as proper stewardship of glyphosate and GT crops is ignored.

Glyphosate Resistance Mechanisms in Weed Species

Several different mechanisms have been discovered in weeds that allow them to withstand doses of glyphosate previously known to control them. These mechanisms can be grouped into two broad categories: Non-target-site and target-site based. Non-target-site resistance to glyphosate involves physiological and biochemical processes that prevent the herbicide from reaching its intended site of action, while target-site based resistance involves changes to the site-of-action of glyphosate, i.e. EPSPS gene.

Non-target-site Based Glyphosate Resistance

Examples of non-target-site based glyphosate resistance include: a) reduced or differential uptake and translocation of glyphosate to its target site, b) rapid sequestration of glyphosate into vacuoles of plant cells, c) rapid necrosis of glyphosate treated tissue, and d) metabolism of glyphosate. Plants with impaired glyphosate uptake grow leaves at angles, or develop thickened cuticles that are not conducive to glyphosate retention or diffusion on the leaf surface, as reported in Italian ryegrass, jackbean (*Canavalia ensiformis*), Johnsongrass

(*Sorghum halepense*), waterhemp (*Amaranthus tuberculatus*) (Chatham 2014; Cruz-Hipolito *et al.* 2009; Michitte *et al.* 2007; Nandula *et al.* 2008; Vila-Aiub *et al.* 2012). Sammons and Gaines (2014) report that reduced translocation of glyphosate result from prevention of glyphosate uptake into the phloem, and retention of the chemical in the tips of treated leaves, keeping it from reaching the meristem or roots. This resistance mechanism is widespread, and has been seen in species such as goosegrass (*Eleusine indica*) (Pline-Srnic 2006), hairy fleabane (Dinelli *et al.* 2008), horseweed (Dinelli *et al.* 2006; Gonzalez-Torralva *et al.* 2012, Nandula *et al.* 2005, Powles and Preston 2006), Italian ryegrass (Nandula *et al.* 2008, Perez-Jones *et al.* 2007), jackbean (Cruz-Hipolito *et al.* 2009), Johnsongrass (Riar *et al.* 2011; Vila-Aiub *et al.* 2012), sourgrass (Gonzalez-Torralva *et al.* 2012), rigid ryegrass (Lorraine-Colwill *et al.* 2002; Nandula *et al.* 2005; Powles and Preston 2006; Wakelin *et al.* 2004), and waterhemp (Chatham 2014). Dinelli *et al.* (2008), Lorraine-Colwill *et al.* (1999), and Shaner (2009, *et al.* 2009). This reduction in glyphosate translocation could be due to several changes, such as the active transport systems of cells no longer recognizing glyphosate, or active pumping of glyphosate out of the chloroplast or cell into the cytoplasm or apoplastic space. Inheritance studies state this trait is controlled by a single, semi-dominant or dominant nuclear gene, and that resistance to glyphosate can be managed by treatment of plants at cooler temperatures (Preston and Wakelin 2008; Shaner 2009; Vila-Aiub *et al.* 2013).

Another mechanism that is closely related, and may work in tandem with reduced translocation, is rapid vacuolar sequestration of glyphosate via a tonoplast transporter, reducing cytoplasmic glyphosate concentrations (Gaines and Sammson 2014; Ge *et al.* 2010, 2011, 2012, Shaner *et al.* 2012). ^{31}P nuclear magnetic resonance spectroscopy research conducted in the

previously mentioned studies found increased selective sequestration of glyphosate in cells in plants such as horseweed, Italian ryegrass, and rigid ryegrass.

The third non-target-site mechanism of resistance is rapid necrosis of glyphosate-treated tissue. This novel form of resistance has only been seen in giant ragweed (*Ambrosia trifida*) (Roberston 2010; Segobye 2013). Glyphosate resistance is imparted by hypersensitive response within 12 hours of treatment. However, it does not seem to be related to changes in salicylic and/or jasmonic acid levels, as the levels of these compounds were not different between resistant and susceptible plants. The rapid cell mortality that occurs reduces the amount of glyphosate translocation from the site of uptake towards meristems, and allows the plants to regenerate and survive (Robertson 2010, Segobye 2013).

The final non-target site based resistance to glyphosate is metabolism of this herbicide. Resistance to glyphosate is caused when biotypes in the population are able to break down glyphosate into less toxic chemicals. Gonzalez-Torralva *et al.* (2012) studied GR horseweed populations from Spain that seemed to metabolize glyphosate into glyoxylate, sarcosine, and aminomethlyphosphonic acid within 96 hours after treatment with the herbicide, whereas glyphosate-susceptible horseweed was only able to metabolize the chemical into glyoxylate in the same time frame. GR sourgrass from Brazil was found to have degraded 90% of the absorbed glyphosate into aminomethylphosphonic acid, glyoxylate, and sarcosine, while susceptible plants only degraded 11% of the absorbed herbicide by 168 hours after treatment (de Carvalho *et al.* 2012). In both these studies, metabolism was seen in plants that also had other non-target site mechanisms of resistance, such as altered uptake and translocation, and very little evidence of glyphosate metabolism has been seen in other species.

Target-site Based Glyphosate Resistance

Target-site based resistance to glyphosate is bestowed by a genetic or physiological change in the plant at the specific site of action, the EPSPS enzyme, that reduces susceptibility. Mutation in the *EPSPS* gene resulting in substitution of amino acid proline for alanine, leucine, serine, or threonine, causing a change in the binding site of the EPSPS enzyme (Powles and Preston 2006; Sammons and Gaines 2014; Shaner *et al.* 2012). This change in amino acid decreases the size of the binding site, which prevents glyphosate from binding, while still allowing the smaller, endogenous substrate, PEP, to bind. This form of resistance is thought to be rarer than other glyphosate resistance mechanisms such as reduced translocation or rapid sequestration of glyphosate in the vacuole, due to the lower level of resistance it provides. Chatham (2014), Ng *et al.* (2004), and Wakelin and Preston (2006b) found evidence that this resistance trait is controlled by a single, semi-dominant nuclear gene, similar to the inheritance of reduced glyphosate translocation mechanism. The EPSPS gene mutations were documented in GR goosegrass (Baerson *et al.* 2002; Kaundun *et al.* 2008; Lee and Ngim 2000; Nandula *et al.* 2005; Powles and Preston 2006), Italian ryegrass (Collavo and Sattin 2012; Jasieniuk *et al.* 2008; Perez-Jones *et al.*2007), junglerice (Alarcon-Reverte *et al.*2013), rigid ryegrass (Kaundun *et al.* 2011; Powles and Preston 2006; Wakelin and Preston 2006a,b), and tall waterhemp (*Amaranthus tuberculatus* var. *tuberculatus*) (Bell *et al.* 2013). Recently, a biotype of goosegrass was found to be 180 fold more resistant to glyphosate than susceptible plants, due to a double substitution mutation in its *EPSPS* gene (Yu *et al.* 2015). The mutations present are the previously known proline 106 to serine and a naturally evolved threonine 102 to isoleucine (TIPS) mutation. This set of mutations is similar to the first generation of transgenically produced corn tolerant to glyphosate.

The second method of target-site resistance to glyphosate, which is the focus of this thesis, is caused by amplification of *EPSPS* gene copies. Generally, amplification of this gene causes increased expression, elevated enzyme activity, and higher protein content of the gene (Powles *et al.* 2010; Sammons and Gaines 2014). This increase in the *EPSPS* gene copy number causes an excessive amount of the enzyme to be produced, which acts like a sponge, binding and deactivating glyphosate in solution, while the remaining unbound portion EPSPS functions normally, ensuring plant survival. This has become a very widespread mechanism of glyphosate resistance, and screening for elevated *EPSPS* copy number has become normal procedure in evaluating GR weeds. Resistance by elevated *EPSPS* genomic copy number has been observed in many species such as Italian ryegrass (Salas *et al.* 2011), kochia (*Kochia scoparia*) (Godar 2014; Jugulam *et al.* 2014; Niehues 2014; Wiersma *et al.* 2014), Palmer amaranth (Chandi *et al.* 2012; Gaines 2009, *et al.* 2010, 2011, 2013; Giacomini *et al.* 2014; Mohseni-Moghadam *et al.* 2013; Ribeiro *et al.* 2014; Teaster and Hoagland 2014; Vila-Aiub *et al.* 2014; Whitaker *et al.* 2013), and waterhemp (Chatham 2014). This mechanism has been extensively studied, and increased *EPSPS* genomic copy number correlated positively with increased resistance to glyphosate when compared to susceptible plants with lower *EPSPS* copy number.

It has been found that different species require different *EPSPS* copy numbers to confer resistance to glyphosate. For example, Palmer amaranth from Georgia and elsewhere in the United States required 30 to 50 copies of glyphosate to survive a field dose of glyphosate (868 g ae ha^{-1}) and plants with five to 160 copies of *EPSPS* have been found (Gaines *et al.* 2010, 2011). Other species, such as GR Italian ryegrass had up to 25 copies (Salas *et al.* (2012), or kochia that needed 3-10 copies for resistance to field use rate of glyphosate (Niehues, 2014; Jugulam *et al.* (2014); Wiersma *et al.* 2014). Inheritance of resistance also seems to differ among plants, with

inheritance of resistance not following Mendelian segregation reported for Palmer amaranth (Chandi *et al.* 2012;Gaines 2009, *et al.* 2010, 2011, 2013; Mohseni-Moghadam *et al.* 2013), while in kochia it follows a Mendelian single-gene inheritance pattern (Jugulam *et al.* 2014; Niehues 2014).

The copy number threshold necessary for glyphosate resistance and inheritance are most likely explained by the genetic mechanisms involved in *EPSPS* amplification in these species. Florescence *in situ* hybridization (FISH) of GR Palmer amaranth (Gaines *et al.* 2010) suggests that *EPSPS* copies spread throughout the genome on every single chromosome and they hypothesize that this distribution of EPSPS copies throughout the genome may have been facilitated via transposable elements. This hypothesis was tested in another study by Gaines *et al.* (2013) where they found sequences similar to miniature-repeat transposable elements (MITEs). These sequences were found flanking the gene copies in GR plants. Furthermore, *Activator* (*Ac*) transposases and repetitive sequences associated with transposons were also seen. Although this study does not conclusively suggest the involvement of transposable elements in EPSPS copy distribution, it provides some evidence indicating possible transposon activity with the duplication and insertion of this gene throughout the Palmer amaranth genome. Another factor affecting the peculiar inheritance of glyphosate resistance in Palmer amaranth was studied in Rebeiro *et al.* (2014) where inheritance of resistance could be affected by Palmer amaranth females to reproducing by apomixes in the absence of suitable pollen, which likely increases stability and inheritance of the resistance trait. An example of transposons containing fully functioning genes capable of expression was seen *Arabidopsis.* Hoen *et al.* (2006) described functional genes and pseudogenes in transposons that are typically silenced by small RNAs. However, in the absence of these RNAs, the genes are expressed and produce their gene product.

While not similar to the situation seen in Palmer amaranth, it does show the possibility of transient genes in transposons retaining their original function, even without permanent reinsertion into the genome. FISH results from kochia indicate all the amplified *EPSPS* copies to be located on two homologous chromosomes, and these copies are distributed in tandem on these chromosomes as discovered by fiber FISH (Jugulam *et al.* 2014). This may have been caused as a result of unequal crossing over between the homologous chromosomes.

Additionally, EPSPS gene duplication was found to be caused by amplification of only one of two *EPSPS* alleles (Gaines *et al.* 2013; Wiersma *et al.* 2014). The other allele only appears in susceptible plants. No evidence of alternative splicing of the *EPSPS* gene has been seen, and no other genes seem to have their expression reduced as a result of elevated *EPSPS* copy number. GR plants that carry this mechanism also show no signs of a fitness penalty that would be thought to accompany such increases in gene copy number and expression. Plants with increased *EPSPS* copy number and expression were found to grow and reproduce similar to susceptible plants, so this trait will most likely persist in the absence of selection by glyphosate (Giacomini *et al.* 2014; Vila-Aiub *et al.* 2014).

Gene amplification conferring resistance to chemicals is not limited to plants, and actually has been studied extensively in arthropods and eukaryotic cancer cells (Powles *et al.* 2010). Bass and Field (2011) report that pesticide resistance in arthropods is typically the effect of one of two mechanisms: increased production of metabolic enzymes that detoxify or inactivate a wide variety of pesticides via amplification of the esterase, glutathione S-transferases, or cytochrome P450 monooxygenase encoding genes, and changes in the protein target due to mutation that leads to a decrease in pesticide sensitivity. Peach-potato aphids (*Myzus persicae*) were found to be resistant to many different insecticides due to amplification of

the metabolism gene esterase E4, and the level of resistance correlated with the increase in esterase activity from gene amplification (Field *et al.* 1988; Field and Devonshire 1997). *Culex* mosquitoes became resistant to organophosphorus compounds when esterase B1 and B2 metabolism genes were amplified up to 250 times more copies in resistant biotypes compared to susceptible ones (Mouches *et al.* 1986; Paton *et al.* 2000: Raymond *et al.* 1993). Resistance also seemed be linked to homozygosity of the amplified genes, and finding heterozygous individuals with lower copy numbers and resistance level was uncommon. One example in human cancer cells was Schimke (1986) where cancer cells with resistance to the chemotherapy drug methotrexate was due to an increased production of the dihydrofolate reductase enzyme, the cause was increased copy number of the encoding gene. Another example of drug resistance in human cancer cells was due to amplification of the gene encoding the MET receptor, which is important for wound healing, caused resistance to epidermal growth factor receptor (EFGR) tyrosine kinase inhibitors in lung cancer cells. (Turke *et al.* 2010). This drug is typically effective at combatting the cancer cells, however, resistance typically develops, but found to be reversible *in vivo* by inhibition of MET and EGFR structural genes. These examples set the basis for the research presented here which focuses on the study of glyphosate-resistance due to *EPSPS* gene amplification in common waterhemp from Kansas.

Common Waterhemp

Habitat and Biology of Common Waterhemp

Common waterhemp (*Amaranthus tuberculatus* var. *rudis*) is an annual herbaceous weed native to North America (Trucco and Tranel 2011). This species has a taproot and mature plants have erect stems and grow to be 2-3 meters tall (Costea *et al.* 2005; Steckel 2007). Trucco and Tranel (2011) state that their stems branch out into terminal inflorescences that have linear spikes

as well as panicles. They have little pubescence on their leaves and stems. Their leaves are ovate, rhombic-oblong to lanceolate-oblong in shape which are 2-10 centimeters long by 1-3 centimeters wide, and have long petioles. This species germinates and emerges in late spring and is continuous, with plants emerging throughout the summer season (Costea *et al.* 2005; Steckel 2007). It has been historically found to grow along fresh water margins near ponds and riverbanks, as well as disturbed areas, preferring well-drained, high-nutrient soils, however waterhemp can survive and thrive in many environments and climates (Costea *et al.* 2005). It has aC4 photosynthetic pathway, as well as Kranz anatomy in its leaves, adaptions that have aided in the colonizing a multitude of regions. Costea *et al.* (2005) notes that it has high photosynthetic rates at high temperatures and light levels and can grow very fast, and has reduced tendency for photorespiration. It does not tolerate saline soils, but can survive brief flooding events leading to anoxic soils and is tolerant of high levels of calcium carbonate. This predilection for areas with high nutrient content and photosynthetic adaptations are reasons it is a major weed species in the Midwest, and is found in a total of 35 states, as well as Canada and Europe (Costea *et al.* 2005).

Common Waterhemp Reproduction and Taxonomy

Waterhemp is dioecious in nature, with separate female and male plants, and only propagates by seeds (Costea *et al.* 2005). They are facultative short-day flowering plants, and initiation of flowers is dependent on day length. Later emerging plants initiate flowering earlier, requiring 14 to 16 eight hour days for reproductive initiation. This however, also potentially decreases their vegetative growth as well as seed production. Costea *et al.* (2005) states plants that emerge earlier will take advantage of the longer days and growing season, increasing their vegetative growth and needing 16 hour long days to flower. These plants will produce more seed, with an average female capable of producing between 35,000 to 1,200,000 seeds,

depending on emergence date and light level (Costea *et al.* 2005; Steckel 2007). Sellers *et al.* 2003 states that out of all the amaranth species, waterhemp produces the most seeds per plant weight. Seeds are spread by wind, water, animals, and farming equipment, and initially have 80% viability, which decreases over time and depth of burial. Costea *et al.* (2005) states its pollen has allergenic properties, and can travel long distances and retain viability. This species is diploid (2n=32 chromosomes), and due to its tremendous geographical range and dioecious nature, the species immense genotypic and phenotypic variation (Costea *et al.* 2005).

Common waterhemp is a member of the Amaranthaceae family, and the subgenus *Acnida* (Costea *et al.* 2005). Pratt and Clark 2001 indicate that common waterhemp and tall waterhemp (*Amaranthus tuberculatus* var, *tuberculatus*) were once considered separate species, however, genetic and morphological analysis concluded that they were in fact regional extremes of a single species, and were combined as such, with two separate varieties *A. tuberculatus* var. *rudis* for common waterhemp, and *A. tuberculatus* var. *tuberculatus* for tall waterhemp. Common waterhemp was traditionally seen growing west of the Mississippi river from Nebraska to Texas, and tall waterhemp east of the Mississippi from Indiana to Ohio (Steckel 2007). However, common waterhemp's geographical range moved increasingly northward, into tall waterhemp's natural range (Trucco and Tranel 2011). Numerous hybridization events were observed between the two, another factor in their grouping as a single species, as it had become impossible to indicate a sure distinction of one species from the other (Steckel 2007; Trucco and Tranel 2011). It should be noted, however, that historically, common waterhemp had greater weedy tendencies than tall waterhemp, and the weed complex has become a major issue due to their exceptional ability to acquire or evolve resistance to herbicides, as will be discussed further.

Waterhemp's ability to hybridize with other amaranths, including monoecious species, is a concern to weed scientists (Costea *et al.* 2005). The main reason for this, as stated in Tranel *et al.* (2002), is that amaranth species coexisting in an area could lead to genetic transfer between these different species, including herbicide resistance genes, and extensive research has been devoted to discovering the potential likelihood of this occurring. A study by Frannsen *et al.* (2001b) crossed acetolactate synthase (ALS)–inhibitor resistant Palmer amaranth (*Amaranthus palmeri*) with susceptible common waterhemp and found evidence of low levels of recombination between the species, including resistance gene transfer. Wetzel *et al.* (1999b) showed the morphology of hybrids made between Palmer amaranth and common waterhemp was dependent on the female, with hybrids derived from crosses with female *A. palmeri* plants showing maternal morphology, and hybrids from crosses with female common waterhemp showing *A. tuberculatus* maternal morphology. Of note, only 15 hybrids were produced from the 10,000 crosses made in this study. Hybridization frequency between *A.palmeri* and *A. tuberculatus* was 0.08 and 0.19% for two accessions, as seen in Gaines *et al.* (2011). However, Trucco *et al.* (2007) state that Palmer amaranth and common waterhemp crosses are likely to be rare, with high amounts of non-hybrid progeny created by female Palmer amaranth plants by apomixis in the absence of suitable pollen. Crosses between monoecious smooth pigweed (*Amaranthus hybridus*) with ALS resistance and susceptible waterhemp have been conducted, and found that while low in frequency, crosses can occur in both the greenhouse and field (Tranel *et al.* 2002; Trucco *et al.* 2005a,b). Fertile progeny recovered were always dioecious females, most likely due to the sex determination genes of waterhemp. Trucco *et al.* (2009) also found that gene introgression was also one way from *A. hybridus* to *A. tuberculatus*, with any

introgression in the other direction resulting in significant genetic aberration and fitness penalties.

Agricultural Impact of Common Waterhemp

As stated previously, waterhemp is a major weed affecting agricultural systems all over North America, especially in corn and soybean fields in the Midwest (Steckel 2007). Presence of this weed can reduce crop yields to a large extent, if not removed in a timely fashion. A study by Hager *et al.* (2002) showed that if common waterhemp was not removed within two weeks of an Illinois soybean crop growing expanded unifoliate leaves, yield loss would occur, and interference by the weed up to ten weeks after unifoliate expansion could cause up to 43% yield loss over a 3 year period. Corn is also heavily affected, with season-long interference by the waterhemp leading to competition for water and nitrogen in Illinois fields causing up to 74% yield reduction over 2 years, and to preserve maximum yield, waterhemp should be controlled by the V6 growth stage (Steckel *et al.* 2004). Kansas soybean fields can also suffer drastic yield loss from waterhemp, which depending on the year and location can be between 27 to 63% without adequate control (Costea *et al.* 2005).

Herbicide Control of Common Waterhemp

Given the time sensitive nature in controlling waterhemp growth, and the evolution of herbicide resistance, managing waterhemp is a daunting task. Costea *et al.* (2005) notes that preplant chemical control of waterhemp is not typically effective unless it has residual control, as the weed emerges after most crop species. Sulfentrazone is found to be a very effective soil-applied herbicide for waterhemp in soybeans, with up to 98% control of plants in the field. Flumioxazin, dimenthamid, S-metolachlor, pendimethalin, acetochlor, linuron, imazethapyr, metribuzin, flufenacet, flumetsulam (with metribuzin or metolachlor) also provide adequate pre-

emergence control, and must be applied as close to crop planting as able to prolong activity into the waterhemp growing season (Costea *et al.* 2005). Post-emergence herbicides can also be effective in controlling this weed, however, due to its continuous emergence pattern, multiple applications will be necessary for optimum control. Effective control of waterhemp plants 10 cm in height can be facilitated with herbicides such as lactofen, fomesafen, acifluorfen, imazamox, imazethapyr, chlorimuron, and thifensulfuron.

Pre-emergence control of waterhemp in corn can be achieved using S-metolochlor and atrazine, as well as pendimethalin, dimethenamid, isoxaflutole, and mesotrione, with multiple applications including atrazine being most effective, however, atrazine resistance is also becoming widespread in this weed species (Costea *et al.* 2005). Waterhemp control after emergence can be accomplished with atrazine, primisulfuron, prosulfuron and dicamba, 2,4-D with atrazine, diflufenzopyr and dicamba, and mesotrione. Glufosinate or glyphosate resistant crops are also an effective management tool for waterhemp control, but due to a lack of residual activity of these herbicides, multiple applications would be necessary for adequate season long control. Also, as mentioned earlier, herbicide resistance to multiple modes of action are present and widespread in waterhemp, severely limiting control options (Costea *et al.* 2005).

Evolution of Herbicide Resistance in Common Waterhemp

The extensive genetic diversity and dioecious nature of waterhemp facilitated evolution of resistance to six different herbicide modes-of-action: *acetolactate synthase* (*ALS*) inhibitors, photosystem II (PSII) inhibitors, *5-enolpyurvylshikimate-3-phosphate synthase* (*EPSPS*) inhibitors, *protoporphyrinogen oxidase* (*PPO*) inhibitors, *4-hydroxyphenylpyruvate dioxygenase* (*HPPD*) inhibitors, and synthetic auxins as of February 2015 (Heap 2015). Examples *ALS* inhibitor-resistant waterhemp is reported to have cross-resistance to flumetsulam (Foes *et al.*

1998), imazamox (Bell *et al.* 2013; Patzoldt *et al.* 2006), imazethapyr, (Foes *et al.* 1998; Horak and Peterson 1995; Sprague *et al.* 1997), and thifensulfuron (Foes *et al.* 1998; Horak and Peterson 1995; Patzoldt *et al.* 2006). This is caused by an insensitive *ALS* enzyme mutant containing a leucine 569 tryptophan substitution in the structural gene. Waterhemp is also resistant to the PSII- inhibitor, atrazine (Bell *et al.* 2013; Foes *et al.* 1998; Patzoldt *et al.* 2005) due to a mutation causing glycine to serine substitution at residue 264 in the D1 protein, or metabolism of the herbicide. *PPO* inhibitor resistance is thought to be unique in that it is caused by a codon deletion and inheritance is controlled by a single, incompletely dominant nuclear gene (Patzoldt *et al.* 2006a). Patzoldt *et al.* (2005) reported a strain of waterhemp that was resistant to the *PPO* inhibiting herbicides acifluorfen, fomesafen, and lactofen. Resistance to lactofen was also reported by Bell *et al.* (2013). *HPPD* inhibitor (e.g. mesotrione) resistance was studied by Hausman *et al.* (2010) and Ma *et al.* (2013). Mesotrione-resistant plants metabolized the mesotrione faster than susceptible waterhemp plants (Ma *et al*., 2013). Waterhemp plants resistant to the synthetic auxin 2,4-D, likely due to increased metabolism, have been reported on the International Survey of Herbicide Resistant Weeds (Heap 2015; Leibhart *et al.* 2014a,b).

Even though waterhemp's resistance to a variety of herbicides has been reported, resistance to glyphosate remains the most pressing mechanism to investigate and understand, given the prevalence of glyphosate use in world-wide agricultural practices, and establishes the significance for the research summarized herein. Previous work on glyphosate resistance in waterhemp by Bell *et al.* (2013) found a proline to serine substitution in EPSPS in a population of waterhemp that is resistant to multiple modes of action of herbicides. Two populations that were 19 and 9 times more resistant to glyphosate, respectively, were found in Missouri, and were also not adequately managed by both *ALS* and *PPO* inhibitors (Legleiter and Bradley 2008). A

Texas population of common waterhemp resistant to glyphosate, 3.5 to 59.7 times the field dose of glyphosate (868 g ae ha^{-1}) was required to cause 50% mortality (Light *et al.*2011). Nandula *et al.* (2013) worked with a Mississippi population of waterhemp that was five times more resistant to glyphosate than a susceptible biotype, and resistance was caused by both reduced uptake of glyphosate as well as the proline to serine substitution in the EPSPS gene. All GR populations from IL studied by Tranel *et al.* (2010) was resistant to *ALS* inhibitors, and 40% were resistant to *PPO* inhibitors as well. Chatham (2014) studied glyphosate resistant in waterhemp extensively, and found a Kentucky population resistant due to the proline 106 to serine substitution in the EPSPS gene, but populations from Illinois, Kansas, Missouri, and Nebraska all were resistant due to *EPSPS* gene amplification. It is important to understand the overall mechanism of glyphosate resistance in this weed, as this could lead to solutions for control of GR biotypes. As such, the goals of this research project which was to determine the relationship between *EPSPS* genomic copies, EPSPS expression level and level of glyphosate resistance, as well as characterizing the distribution and configuration of *EPSPS* copies in glyphosate resistant waterhemp from Kansas.

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Figures

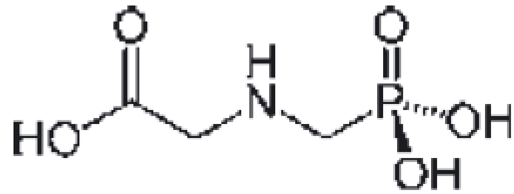

Figure 1.1: The structure of glyphosate. Adapted from Dill *et al.*(2010).

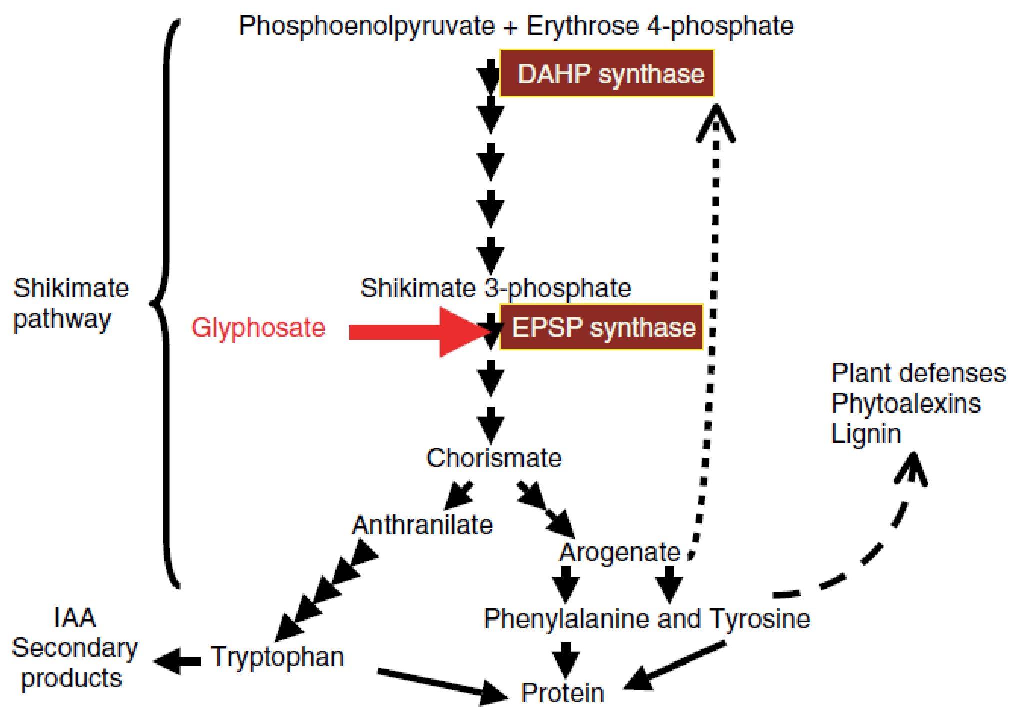

Figure 1.2: The shikimate pathway and the site of inhibition by glyphosate. Products of the pathway and regulatory feedback inhibition (dotted arrow) are shown. Adapted from Duke and Powles (2008).

Chapter 2 - Relationship between the Level of Glyphosate Resistance, *EPSPS* Copy Number, and Expression in Common Waterhemp (*Amaranthus rudis*) from Kansas

Abstract

Common waterhemp (*Amaranthus rudis*) is a problematic weed species of cropping systems throughout the Midwestern states, including Kansas. Recently, waterhemp populations from Kansas were found to have evolved resistance to the widely used herbicide glyphosate as a result of amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS)*, the enzyme target of glyphosate. The objectives of this research were to 1) perform glyphosate dose-response study and determine the relationship between relative *EPSPS* genomic copies and *EPSPS* gene expression in glyphosate-resistant waterhemp, and 2) characterize the genomic configuration and distribution of *EPSPS* copies using florescence in situ hybridization (FISH) in three glyphosate-resistant waterhemp populations. Waterhemp populations from eastern Kansas were screened with 868 g ae ha^{-1} (field used rate) of glyphosate, and genomic DNA and total RNA was isolated from the survivors to determine the *EPSPS* genomic copies and *EPSPS* gene expression relative to the *acetolactate synthase* (*ALS*) gene using qPCR. Furthermore, waterhemp specific *EPSPS* probes were synthesized to perform florescence in situ hybridization (FISH) on these glyphosate-resistant plants. Results of these experiments indicate a positive correlation between level of glyphosate resistance, *EPSPS* copies, and their expression. As expected, a negative correlation was found between shikimate accumulation and *EPSPS* copies. Sequencing of the *EPSPS* gene showed no presence of the proline 106 mutation, which is known to be associated with glyphosate resistance suggesting that an insensitive *EPSPS* enzyme was not involved in the mechanism of glyphosate resistance. FISH analysis of resistant plants illustrated

presence of amplified *EPSPS* copies on two homologous chromosomes, likely near the centromeric region. . This is the first report demonstrating a positive relationship between *EPSPS* copies and expressions, as well as chromosome configuration of *EPSPS* copies in glyphosate-resistant waterhemp from Kansas.

Introduction

Common waterhemp (*Amaranthus tuberculatus* var. *rudis*) is a major weed of cropping systems in North America (Trucco and Tranel 2011). This weed is especially problematic in agricultural fields of the Midwestern states, reducing yields in Illinois corn fields up to 74%, and causing yield losses of 23 to 63% in Illinois and Kansas soybean fields (Costea *et al.* 2005; Hager *et al.* 2002; Steckel *et al.* 2004, 2007). This species is well adapted to diverse environments (Costea *et al.* 2005). Common waterhemp is dioecious in nature, and a prolific seed producer, as a single female plant can produce up to 35,000 to 1,200,000 seeds (Costea *et al.* 2005). Because of availability of extensive genetic variability, coupled with extensive herbicide selection pressure, waterhemp evolved resistance to several herbicides with different modes of action. Currently this species evolved resistance to six different herbicide modes of action, including *acetolactate synthase* (*ALS*), photosystem II*, 5-enolpyruvylshikimate-3-phosphate synthase*, *protoporphoryninogen oxidase* (*PPO*),and *4-hydroxyphenylpyruvate dioxygenase* (*HPPD*) inhibitors, as well as synthetic auxins (Heap 2015).

Glyphosate (N-(phosphonomethyl) glycine), the most widely used herbicide around the globe was first commercialized by the Monsanto Company in the 1970s as the product Roundup (Dill *et al.* 2010; Duke and Powles 2008; Alibhai and Stallings 2001). It is currently the most popular herbicide due to its inherent safety and effectiveness in controlling weeds (Alibhai and Stallings 2001; Healy-Fried *et al.* 2007; Pline-Srnic 2006). Glyphosate works by inhibiting the 5-enopyruvlyshikimate-3-phosphate synthase (EPSPS) enzyme of the shikimate pathway, preventing the synthesis of the aromatic amino acids tyrosine, tryptophan, and phenylalanine (Steinrucken and Amrhein 1980, Amrhein *et al.* 1980; Duke and Powles 2008). This herbicide is also unique in that it only affects the EPSPS enzyme with no known off target effects.

Extensive and exclusive use of glyphosate over a prolonged period resulted in incredible selection pressure on weed species leading to evolution of glyphosate resistance in a number of weed species, such as rigid ryegrass (*Lolium rigidum* Gaudin.) (Feng *et al.* 1999; Lorraine-Colwill *et al.* 2001; Pederson *et al.* 2007; Powles *et al.* 1998; Pratley *et al.* 1999; Simarmata *et al.* 2003, 2005), horseweed (*Conyza canadensis* L. Cronq.) (Koger *et al.* 2004; Main *et al.*2004; Mueller *et al.* 2003; VanGessel *et al.* 2001; Zelaya *et al.* 2004), and Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Culpepper *et al.*2006; Norsworthy *et al.* 2008, Steckel *et al.* 2008; Teaster and Hoagland 2013) and kochia (*Kochia scoparia* (L.) Schrad) (Wiersma, et al., 2014; Godar et al., 2014). According to Heap (2015), there are currently 32 glyphosate-resistant weeds worldwide in 22 different countries.

The known mechanisms of glyphosate resistance in weed species can be grouped into two broad categories: non-target-site and target-site resistance. Non-target-site resistance mechanisms include reduced translocation or sequestration of glyphosate as reported in horseweed and rigid ryegrass (Dinelli *et al.* 2006; Gonzales-Torralva *et al.* 2012; Nandula *et al.* 2005; Powles and Preston 2006; Lorraine-Colwill *et al.* 2006; Nandula *et al.* 2005; Powles and Preston 2006; Wakelin *et al.* 2004). Target-site resistance to glyphosate is generally evolved as a result of mutation in *EPSPS* gene, at proline 106 to alanine, leucine, serine, or threonine as documented in goosegrass (*Eleusine indica* L. Gaertn;(Baerson *et al.* 2002; Kaundun *et al.* 2008; Lee and Ngim 2000; Nandula *et al.* 2005; Preston and Powles 2006) and rigid ryegrass (Kaundun *et al.* 2011; Powles and Preston 2006; Wakelin and Preston 2006a,b). A population of glyphosate-resistant goosegrass was found to have double mutations at threonine 102 to isoleucine and proline 106 to serine (TIPS) (Yu *et al.* 2015). This double mutation results in a greater level of glyphosate resistance than what is seen with the P106 mutation alone. Another

target-site glyphosate resistance mechanism, i.e., amplification of the *EPSPS* structural gene was more recently reported in several weed species. This mechanism was first discovered in *A. palmeri* (Gaines *et al.* 2010), however it has been shown in other species including *Kochia scoparia* (Godar 2014; Jugulam *et al.* 2014; Niehues 2014; Wiersma *et al.* 2014), waterhemp (*A. tuberculatus* var. *tuberculatus*/*rudis*) (Chatham 2014) and Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) (Salas *et al.* 2012). Sammons and Gaines (2014) and Powles *et al.* (2010) state that increased genomic copy number of this gene leads to increased enzyme expression allowing the plant to survive normally lethal applications of glyphosate. Gene amplification endowing resistance to insecticides has also been reported in arthropod species such as *Myzus* aphids and *Culex* mosquitoes, as well as in drug resistant cancer cells (Bass and Field 2011; Field *et al.* 1988; Field and Devonshire 1997; Mouches *et al.* 1986; Paton *et al.* 2000; Poules *et al.* 2010; Raymond *et al.* 1993; Turke *et al.* 2010).

Gaines *et al.* (2010, 2013) hypothesizes that in *A. palmeri* amplification of the *EPSPS* gene may have facilitated by mobile transposable elements as duplicated copies are distributed throughout the genome. Later work discovered sequences flanking the amplified *EPSPS* copies that were similar to those found in miniature inverted-repeat transposable elements (MITEs), and *Acitivator* (*Ac*) transposase and repetitive sequence regions (Gaines *et al.* 2013). However, in *K.scoparia*, tandem arrangement of duplicated copies of *EPSPS*, possibly because of unequal recombination between two homologous chromosomes was reported (Niehues 2014 and Jugulam *et al.* 2014). Likewise, Chatham (2014) showed amplification of the *EPSPS* gene involvement in glyphosate resistance in waterhemp as well. However, information about *EPSPS* gene expression and the distribution of *EPSPS* gene copies in the genome of waterhemp is not known. Therefore, the purpose of this research was to a) determine the relationship between the level of glyphosate

resistance and *EPSPS* genomic copies, and expression, and b) characterize the chromosomal distribution and configuration of *EPSPS* copies in the genome of glyphosate-resistant common waterhemp from Kansas.

Materials and Methods

Characterization of Glyphosate Resistance in A. rudis

Seeds of three glyphosate-resistant *A. rudis* populations collected from eastern Kansas (Table 2.1), as well as a known susceptible population, from Putman (2013) were planted in 30 x 22 x 6 cm plastic trays filled with Miracle-Gro moisture control potting soil and grown in the greenhouse attached to the Agronomy Department at Kansas State University. The greenhouse was maintained at 25/20 C (day/night) with a 15/9 hour night photoperiod. Sunlight was supplemented with sodium vapor lamps with a 200 µmol $m^{-2}s^{-1}$ photosynthetic flux. When plants were at 8-10 cm tall, about 20 plants per tray were sprayed with either 1x, 2x, or 4x (where x is the field used rate of glyphosate, which is 868 g ae ha^{-1} with 2% ammonium sulfate (AMS) (v/v)) dose of glyphosate (Roundup WeatherMAX, Monsanto Company). This was initially done in spring 2013, and repeated in spring 2014. The *A. rudis* populations known to have low, medium and high level of resistance, were sprayed with 1x, 2x and 4x dose of glyphosate, respectively. In all experiments glyphosate was applied using a track sprayer (Generation III Research Sprayer, De Vries Manufacturing, Hollandale, MN), calibrated to apply 168 L ha^{-1} at 220 kPa with a flat fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL). Two weeks after glyphosate application, the survivors were transplanted to 11 x 11 x 12 cm plastic pots containing Miracle-Gro moisture control potting soil. All plants were watered regularly and Miracle-Gro All Purpose Plant Food (24-8-16) was applied to the plants every two weeks. Leaf tissue from these plants was used to perform shikimate accumulation assays, genomic DNA and

total RNA extraction. The leaf tissue was harvested from these plants and was flash frozen in liquid nitrogen and then stored at -80°C until further use. Furthermore, these plants were also propagated vegetatively by nodal cuttings to perform whole-plant dose response experiments.

Shikimate Assays

As noted previously, in sensitive plants glyphosate inhibits the EPSPS enzyme, causing a reduction in aromatic amino acid synthesis and buildup of shikimic acid (Shaner *et al.* 2005). Quantification of shikimate is often used to measure glyphosate resistance level in plants. Twelve 6 mm leaf discs were harvested from the youngest leaf using a paper hole-punch from both resistant and susceptible plants. These discs were placed in 96-well microtiter plates, with one disc per well. The wells were organized in four columns of three wells for each treatment (100, 250, and 500 µM gyphosate), and a control well contained only buffer A (0 µM glyphosate, 0.6902 ammonium phosphate dissolved in 600 ml deionized water). The plates were wrapped in clear plastic wrap and incubated under artificial light (200 mmol $m^{-2}s^{-1}$) for 16 hours. Following this step, the plates were placed in a -20 freezer for 30 minutes and then thawed for 20 minutes in a 60 C oven. The discs were subsequently treated with 25 µL 1.25 N HCl and incubated again at 60 C for 20 minutes, until the tissue turned grey-brown. The 25 µL of the solution in each of the original wells was transferred into new 96-well microtiter plates containing 100 µL reaction buffer (periodic acid, 0.25% v/v, meta-periodate, 0.255 v/v) and incubated for 20 minutes at 40 C. Subsequently, 100 µL of quench buffer (0.6 M sodium hydroxide, 0.22 M sodium sulfite) was added to each well. Using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc.) with Gen5 version 2.01 software, shikimate concentration was measured at $OD_{380.}$ A shikimate standard curve was generated, and used to determine the concentration of shikimate in ng μL^{-1} in each well. Shikimate concentration data

were analyzed in SAS version 9.4 using PROC GLM, and least significant difference (LSD) was measured using least square means (LSMEAN), and contrasts.

Determination of Relative EPSPS Copies

In order to determine the relative *EPSPS* copies of *A. rudis* plants, genomic DNA (gDNA) was extracted using approximately 100 mg of fresh leaf tissue of resistant and susceptible plants. This tissue was collected and placed into 1.5 ml microcentrifuge tubes, and flash frozen in liquid nitrogen. The tissue was then disrupted in the microcentrifuge tubes, using plastic pestles, and gDNA was extracted using a Qiagen DNeasy Plant Mini Kit (Cat. No. 69104) according to the protocol supplied by the manufacturer. The gDNA was diluted to 8 ng/µL with molecular grade water (RNase/DNase/protease Free) from G Biosciences. The gDNA concentration of each sample was quantified using a NanoDrop® 1000 spectrophotometer (Thermo Scientific). The *ALS* gene was used as a reference gene to determine the relative *EPSPS* copy number, according to Gaines *et al.*(2010). Quantitative PCR was conducted using a Bio-Rad CFX-96 Touch unless otherwise noted. *A. palmeri* primers for both *ALS* (forward: 5'-GCTGCTGAAGGCTACGCT-3'; reverse: 5'-GCGGGACTGAGTCAAGAAGTG-3'; 118 base pair product) and *EPSPS* (forward: 5'ATGTTGGACGCTCTCAGAACTCTTGGT-3'; reverse: 5'-TGAATTTCCTCCAGCAACGGCAA-3'; 195 base pair product) synthesized by Gaines *et al.* (2010) were used in all qPCR experiments conducted using gDNA. Each reaction well in a 96-well microtiter plate contained 10 µL of iQ™ SYBR® Green Super Mix (Bio-Rad), 2 µL each of forward and reverse primers (5 µM; *ALS* or *EPSPS*, depending on the well), 2 µL of 16 ng/µL gDNA, and 4 µL DNase/RNase/protease molecular grade water (G Biosciences) to a total volume of 20 µL. Reactions with each primer set were done in triplicate and each experiment was conducted at least twice. The following conditions were maintained for PCR: initial

denaturing at 95 C for 15 minutes, followed by up to 40 cycles of denaturing at 95 C for 30 seconds, and then annealing and extension at 60 C for 1 minute. This cycle was repeated 39 more to a total of 40 times per run.

C_T thresholds were used to find the *EPSPS* genomic copy number, which was normalized using the *ALS* reference gene. This was accomplished with the equation ΔC_T = average C_T^{ALS} - C_T^{EPSPS} adapted from Gaines *et al.* (2010). The equation $2^{-\Delta C_T}$ = relative gene copy (ΔC_T method) was used to determine the copy number. Additionally, qPCR was also used to test the stability of *EPSPS* gene copy number in various parts of a single plant.

Determination of Relative EPSPS cDNA Expression

A. rudis EPSPS gene transcript levels were quantified using qPCR and *ALS* as the reference gene as described above for determining the gene copy number. Total RNA from frozen leaf tissue was extracted using a Qiagen RNeasy Plant Mini Kit (Cat. No. 74904) following the manufacturer's instructions. The quantity and quality of total RNA was determined using a NanoDrop® 1000 spectrophotometer (Thermo Scientific) and agarose gel (1%) electrophoresis. RNA was treated with DNase 1 enzyme (Thermo Scientific, Waltham, MA, USA) to remove genomic DNA contamination. cDNA was synthesized from 1 µg of total RNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). Concentration of the cDNA was then determined via NanoDrop® 1000 spectrophotometer (Thermo Scientific), and then used for qPCR as outlined in the previous section.

Whole Plant Glyphosate Dose Response Study

Both resistant and susceptible *A. rudis* plants used in initial screening study were cloned and the clonally propagated plants were used in whole-plant glyphosate dose response experiments. Nodal cuttings of approximately 4-5 cm in size were selected, and the leaves and

petioles were removed. The bottom of the shoot tissue was then treated with Bontone® rooting powder (Bonide Products, Inc., Oriskany, NY) and placed in 6 x 6 x 5 cm plastic pots containing pre-wetted Miracle-Grow Moisture control potting soil. These pots were then covered with a clear plastic humid dome. Once the clonally propagated plants established and reached 8-10 cm in height, at least 2 to 5 clones of each plant were treated with formulated glyphosate (Roundup WeatherMAX, Monsanto Company) with 2% AMS using the previously described. Susceptible and low resistance level plants were treated with 0, 0.625, 0.125, 0.25, 0.5, 1, 2x doses (x = 868 g ae ha^{-1} glyphosate). Moderately and highly resistant plants were treated with 0, 0.25, 0.5, 1, 2, 4x. Plants of highly resistant population were also treated with a 6x dose of glyphosate. Visual injury was recorded at 1, 2 and 3 weeks after treatment (WAT) with glyphosate. Above ground biomass was harvested 3 WAT from all plants, weighed, and dried in an oven at 65 C for 48 hours. The dry biomass was also determined. Data of visual injury rating and plant biomass were analyzed in SAS version 9.4 using PROC GLM, and significant differences was measured using least significant difference (LSD), least square means (LSMEANS), and contrasts. Using the results of the shikimate assays, whole plant glyphosate dose response, as well as *EPSPS* genomic copy number, plants were distributed into categories depending on their resistance level (GS = glyphosate susceptible, GR1 = glyphosate-resistant 1, (low resistance, under two *EPSPS* gene copies), GR2 = glyphosate-resistant 2 (moderate resistance, two to seven *EPSPS* gene copies), GR3 = glyphosate-resistant 3 (high resistance, more than seven *EPSPS* copies)).

Sequencing the EPSPS Gene for Proline 106 Mutations

To determine if known proline 106 mutation(s) are present in EPSPS gene of *A. rudis*, this gene was amplified by PCR using gDNA and *Kochia scoparia* primers from Niehues (2014) (Forward: 5’-CCAAAAGGGCAGTCGTAGAG-3’; reverse: 5’-

ACCTTGAATTTCCTCCAGCA-3’; 200 bp product). These reactions comprised 12.5 µL PCR Master Mix (Promega), 2.5 µL of both forward and reverse primers, 5 µL gDNA (40 ng/µL), and 2.5 µL of nuclease free water (Promega), for a total reaction volume of 25 µL for each sample. The PCR was performed on a T100 Thermal Cycler (Bio-Rad), with the following conditions: initial denaturation at 95 C for 3 minutes, followed by 40 cycles of denaturation at 95 C for 30 seconds, annealing at 53.5 C for 45 seconds and final extension at 72 C for 7 minutes.

Agarose gel electrophoresis was conducted with a 1% agarose matrix stained with ethidium bromide. After expected amplicon size was confirmed using a 100 base pair DNA ladder, the PCR product was then purified using a GeneJET PCR Purification kit (Thermo Scientific #K0701), which was checked on another agarose gel again to confirm the size of the purified product and the band intensity required for sequencing . This product along with one of the primers was sent for sequencing at the Genomic and Sequencing Laboratory, Department of Plant Pathology at Kansas State University. Sequence results were aligned with Multalin software (Corpet 1988).

Distribution and Configuration of Amplified EPSPS Genomic Copies on Common Waterhemp Chromosomes

Florescence *in situ* hybridization (FISH) was performed to illustrate the distribution and configuration of EPSPS copies on chromosomes of *A. rudis* plants. Somatic chromosome preparations (drop technique), using direct probe labeling (nick translation), and the FISH procedure on somatic chromosomes of *A. rudis* plants were performed as described previously (Kato *et al.* 2004, 2006) with minor modifications. Specifically, root tips were collected from young plants (glyphosate-resistant and –susceptible) and treated in a nitrous oxide gas chamber for 2 hours, fixed on ice in cold 90% acetic acid for 10 min, washed and stored in 70% ethanol at

−20 C. For slide preparation, roots were washed in tap water for 10 min and then in KCl buffer for 5 min (75 mM KCl, 7.5 mM EDTA, pH 4); 3-5 meristems (0.5–1 mm long) were placed in 20 µl of 4% cellulase Onozuka R-10 (Yakult, Japan, Tokyo Cat. No. 201069), 1% pectolyase Y23 (Karlan Cat. No. 8006) in KCl buffer, and incubated for 50 min at 37 C. Digested root meristems were washed for 5 min in ice-cold Tris–EDTA buffer, pH 7.6, then three times in 100% ethanol. Meristems were dispersed with a needle in 10-15 µl of ice-cold acetic acid: methanol mix (9:1) and immediately dropped on to two-three pre-cleaned glass slides placed in a humid chamber. Dried preparations were UV cross-linked, soaked in methacarn solution (methanol: chloroform: glacial acetic acid at 6:3:1) for 2 min, dried and used for hybridization on the same day.

For labeling the nucleolus organizing region (NOR) rRNA loci, clone pTa71, containing a 9-kb insertion with 18S, 5.8S, and 26S rRNA wheat genes and intergenic spacers (Gerlach and Bedbrook 1979) was used as a probe. 5 µl of probe mixture contained 200 ng of *EPSPS* PCR primer product-WH2 labeled with Texas red-5-dCTP and 160 ng of pTa71 labeled with Fluorescein-12-dUTP (PerkinElmer, Cat. No. NEL413001EA and NEL426001EA). The mixture of probes (procedure for preparation of probe was given in the next section) and the slide preparation were denatured at 100 C separately. The rest of the FISH procedure and washes were performed by using the methods described by Kato *et al.* (2006). Chromosome preparations were mounted and counterstained with 4', 6-diamidino-2-phenylindole solution (DAPI) in Vectashield (Vector Laboratories, Cat. No. H-1200). FISH images were captured with a Zeiss Axioplan 2 microscope using a cooled charge-coupled device camera CoolSNAP HQ2 (Photometrics) and AxioVision 4.8 software (Zeiss). The final contrast of the images was processed using Adobe Photoshop CS5 software (Adobe Systems Incorporated, San Jose, CA, USA).

Preparation of the EPSPS Probe for FISH

The sequence of *Amaranthus tuberculatus EPSPS* mRNA (GenBank Accession FJ869881) was used to develop the PCR primers (forward: 5'-GCCAAGAAACAAAGCGAAAT-3'; reverse: 5'-TTTCAGCATCATAATTCATAACCC-3'; 1804 base pair product). *A. rudis* RNA was extracted with a Qiagen RNeasy Plant Mini kit (Cat. No. 74904) and total RNA was further treated with DNase I enzyme to eliminated genomic DNA contamination. 1 µg purified RNA was used in first strand cDNA synthesis using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The *EPSPS* cDNA was amplified on a T100 Thermal Cycler (Bio-Rad). The RT-PCR reaction included 40 PCR cycles consisted of 95 C for 3 minutes of initial denaturation, followed by denaturation for 95 C 30 sec, annealing at 53.5 C for 45 seconds, 72 C for 7 minutes for final extension, then the product was held at 4 C. Total reaction volume in each well was 50 µL, with 25 µL PCR Master Mix (Promega), 5 µL forward primers, 5 µL reverse primers, 5 µL (40 ng/µL) cDNA template, and 10 µL nuclease free water (Promega). The PCR products of the expected size were cut and purified from 1% agarose gel stained with ethidium bromide using a Qiagen QIAquick GEL Extraction kit (Cat. No. 28704) and re-amplified using the same primers and protocol, but with a reaction volume per well of 100 µL, comprised of 50 µL PCR MasterMix (Promega), 10 µL forward and reverse primers, 5 µL (40 ng/µL) cDNA template, and 25 µL nuclease free water (Promega). PCR products were purified with Invitrogen PCR Purification kit (Cat. No. K3100-01). The PCR product (WHR2-2) with length ~1.8 kb was used to prepare the FISH probe. This fragment was verified by sequencing with the above primers at the Genomic and Sequencing Laboratory, Department of Plant Pathology at Kansas State University.

Results

Shikimate Concentration, Relative EPSPS Genomic Copy Number, and Relative EPSPS cDNA Expression

Results of the shikimate assays showed greater concentrations of shikimate for all doses in glyphosate-susceptible *A.rudis* plants compared to glyphosate-resistant plants (Figure 2.1). Additionally, all plants showed greater shikimate accumulation with an increase in dose of glyphosate (Figure 2.1). The shikimate concentration also negatively correlated with relative *EPSPS:ALS* genomic copy number (Figure 2.1). Analysis of glyphosate dose response in this assay showed significant difference in shikimate accumulation at 100, 250, and 500 µM of glyphosate among all plants tested.

The relative *EPSPS:ALS* gene copies were determined as follows. The amplicon size of the *ALS* fragment was 118 bp, while the size of the *EPSPS* PCR product was 195 bp. The raw data indicated lower than one relative *EPSPS:ALS* gene copy in most of the glyphosate-susceptible *A. rudis* plants, therefore, for simplicity these values were made equal to one, with the rest of the data normalized according to this. A table 2.2 indicate plants used in this study with their average *EPSPS* copy number and gene expression level (where available). Analysis of relative *EPSPS:ALS* copies suggest that the susceptible *A. rudis* plants had *EPSPS* gene copy values ranging from 0.97 to 1.04 (+/- 0.016) (Figure 2.2). *A. rudis* plants from the population with low glyphosate resistance had average relative *EPSPS:ALS* values of 0.95-1.65 (+/- 0.064) (Figure 2.2). *A. rudis* plants from the moderately glyphosate-resistant population had average relative *EPSPS:ALS* genomic copy number values between 2.0-6.81 (+/- 0.199) (Figure 2.2); whereas *A. rudis* plants from a highly glyphosate-resistant population had between 7.67-16.14 (+/- 0.292) (Figure 2.2) average relative *EPSPS:ALS* genomic copy numbers. Resistance

categories used in the figures (GR1=low, GR2=moderate, and GR3=high) were assigned based on both the level of shikimate concentration (Figure 2.1) and percent visual injury ratings (Figure 2.4), and do not refer to the original populations.

A positive correlation between relative *EPSPS:ALS* gene expression and relative *EPSPS:ALS* genomic copy number was found (Figure 2.3), which indicates that relative *EPSPS:ALS* cDNA expression increases as the number of relative *EPSPS:ALS* genomic copies increases in glyphosate-resistant *A. rudis*.

Glyphosate Dose Response

Results of the whole-plant glyphosate dose response experiments of *A. palmeri* clonal plants showed that 100% injury of susceptible plant and mortality was caused with even 0.0625x glyphosate (1x = 868 g ae ha^{-1}) (Figure 2.4; Figure 2.7 A). GR1 clones typically showed 100% visual injury at 0.125x (Figure 2.4; Figure 2.7 B), however, there were individual clones that survived a dose up to 0.25x. Clones in GR2 generally showed survival up to 2x treatments with 70% visual injury (Figure 2.4; Figure 2.7 C), though individuals did survive up to 4x treatments of glyphosate with over 80% visual injury. GR3 clones showed survival at treatments as high as 6x field rate of glyphosate with approximately 50% visual injury, with mortality evident in only a few individual plants (Figure 2.4; Figure 2.7 D). Clones that survived treatment with glyphosate generally had decreased height and biomass. Overall, there is a clear trend of decreasing susceptibility to glyphosate in plants with increasing *EPSPS* genomic copy number, relative to *ALS* (Figure 2.4; Figure 2.7). For a more in-depth analysis of the glyphosate resistance in the populations used, please refer to Putman (2013).

EPSPS Gene Sequencing

A point mutation of the *EPSPS* gene at residue 106 that replaces proline with alanine, leucine, serine, or threonine has been found to confer glyphosate resistance and prevents glyphosate binding at the target (Powles and Preston 2006; Sammons and Gaines 2014; Shaner *et al.* 2012). This causes the EPSPS enzyme to become insensitive to glyphosate. The *EPSPS* gene of *A. rudis* plants was sequenced to determine if the glyphosate resistance was due to a substitution mutation at residue 106. The results indicate that the residue 106 codes for the amino acid proline in all the *A. rudis* plants sequenced, with no evidence of a point mutation conferring a glyphosate insensitive EPSPS enzyme (Figure 2.5). Therefore, resistance from a substitution mutation of this amino acid is not the cause of glyphosate resistance in the plants studied and suggests that the increased genomic copy number is the primary source of herbicide resistance in these populations of *A. rudis*.

Chromosomal Location and Configuration of EPSPS Genomic Copies in A. rudis

Analysis of FISH results show a marked increase in the visibility and number of *EPSPS* signals in moderately (2-7 copies) and highly (>7 copies) resistant *A. rudis* plants relative to glyphosate-susceptible plants (Figure 2.6). Susceptible and resistant *A. rudis* plants contain their *EPSPS* copies on two homologous chromosome pairs, near pericentric region. In both prophase and metaphase chromosome spreads *EPSPS* gene signal intensity is lower in susceptible plants (Figure 2.6 A, B) than those found in resistant plants (Figure 2.6 C, D), suggesting presence of increased *EPSPS* copies in glyphosate-resistant *A. rudis*. The amplified *EPSPS* gene copies in resistant plants do not appear to be arranged near telomeric region as reported in *Kochia scoparia* (Jugulam *et al.* 2014; Niehues 2014), but instead, are clustered closely near the

centromeric position of the chromosomes. Furthermore, *A. rudis* plants showed *EPSPS* copies only on two homologous chromosomes

Discussion

The data from a variety of experiments including whole plant dose response, shikimate assay, q PCR for *EPSPS* gene copy estimation and expression, as well as FISH analysis, conducted in this research demonstrate that elevated gene copies and expression of *EPSPS* is likely the mechanism of glyphosate resistance in *A. rudis* populations found in Kansas. The level of shikimate accumulation has been shown to correlate negatively with a plant's level of glyphosate resistance (Shaner *et al.* 2005). A multitude of studies have shown a similar relationship, such as in Gaines *et al.* (2010) and Mohseni-Moghadam *et al.* (2013), where susceptible *A. palmeri* was shown to accumulate shikimate after treatment with glyphosate, whereas glyphosate-resistant plants did not, or very minimal accumulation. Nandula *et al.* (2013) and Niehues (2014) showed similar results in tall waterhemp (*A. tuberculatus*) and *K. scoparia*, respectively. Shikimate dose response experiments with doses of 0, 100, 250, and 500 µM glyphosate in this research shows that shikimate concentrations are lower in plants with higher *EPSPS* genomic copy number (Figure 2.4), indicating increased resistance to glyphosate. The lower accumulation of shikimate in glyphosate-resistant *A. rudis* also correlated with increased relative *EPSPS:ALS* genomic copies and glyphosate resistance (Figure 2.4) as reported in glyphosate-resistant *A. palmeri* (Gaines *et al.* 2010, 2011).

The number of relative *EPSPS:ALS* genomic copies required for glyphosate resistance in *A. rudis* plants is similar to what was shown in *K. scoparia*, with around two to three relative *EPSPS* copies providing resistance to a 1x field dose of glyphosate (Jugulam *et al.* 2014; Niehues 2014). On the other hand in *A. palmeri*, roughly 30 to 50 relative *EPSPS* genomic

copies are necessary to survive a 1x dose of glyphosate (Gaines *et al.*2010). It should be noted however, all the plants from the low glyphosate resistance category 'GR1' possess lower than two relative *EPSPS* copies. However, during initial screenings with glyphosate, these plants survived up to a 1x dose of glyphosate. All dose response experiments were conducted on clones, complications of which may have skewed our results (Figure 2.4).

The level of *EPSPS* expression has also been shown to be associated with an increase in glyphosate resistance in *A. palmeri* (Gaines *et al.* 2010, 2011; Mohseni-Moghadam *et al.* 2013). Likewise, a positive correlation between amplified *EPSPS* and the level of *EPSPS* expression in *A. rudis* is seen in this research as well (Figure 2.3). An increase in the *EPSPS* gene copies, as well as expression would lead to an increase in production of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme, allowing plants to tolerate higher dose of glyphosate.

Amplified *EPSPS* copies in these *A. rudis* individuals were located on a pair of homologous chromosomes, similar to what was seen in *K. scoparia* studied in Jugulam *et al.* (2014) and Niehues (2014). However, the amplified *EPSPS* copies in *A. rudis* do not appear to be near the telomeric region on the chromosomes as seen in *K. scoparia*, but instead, are clustered in close proximity, potentially near the centromeres of these chromosomes. While the copy numbers between the glyphosate-resistant *A. palmeri* studied in Gaines *et al.* (2010) and the *A. rudis* studied here are very different, it is possible that the *EPSPS* copies are being amplified and distributed by a similar process in both species: mobile transposable elements. These are autonomous segments of DNA which are mobile in an organism's genome, and often inserting themselves in random positions, and can potentially incorporating functional genes. However, much higher copy number values have been recorded in *A. palmeri* from Gaines *et al.* (2010) than in these *A. rudis* individuals, and later work discovered that these copies are much more

unstable in their insertion through the *A. palmeri* genome, potentially caused by random insertion by transposons (Sammons and Gaines 2014).

The increase in the number of glyphosate-resistant weeds around the worlds is reducing the usefulness of this flexible and historically effective herbicide. Loss of this herbicide could mean use of possibly more expensive or less environmentally friendly herbicides for weed management. Understanding the mechanism of glyphosate resistance in *A. rudis* will help in managing this extremely troublesome weed. Furthermore, this research also uncovers for the first time the genomic distribution of *EPSPS* gene copies on *A. rudis* chromosomes, which will shed light on molecular basis of *EPSPS* gene amplification in *A. rudis*. Knowledge of how this glyphosate resistance mechanism functions and evolves could potentially slow or stop its spread, and give us the tools to preserve the world's most popular herbicide's usefulness into the future, where every option available may be needed to retain and increase our level of agricultural productivity.

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Figures

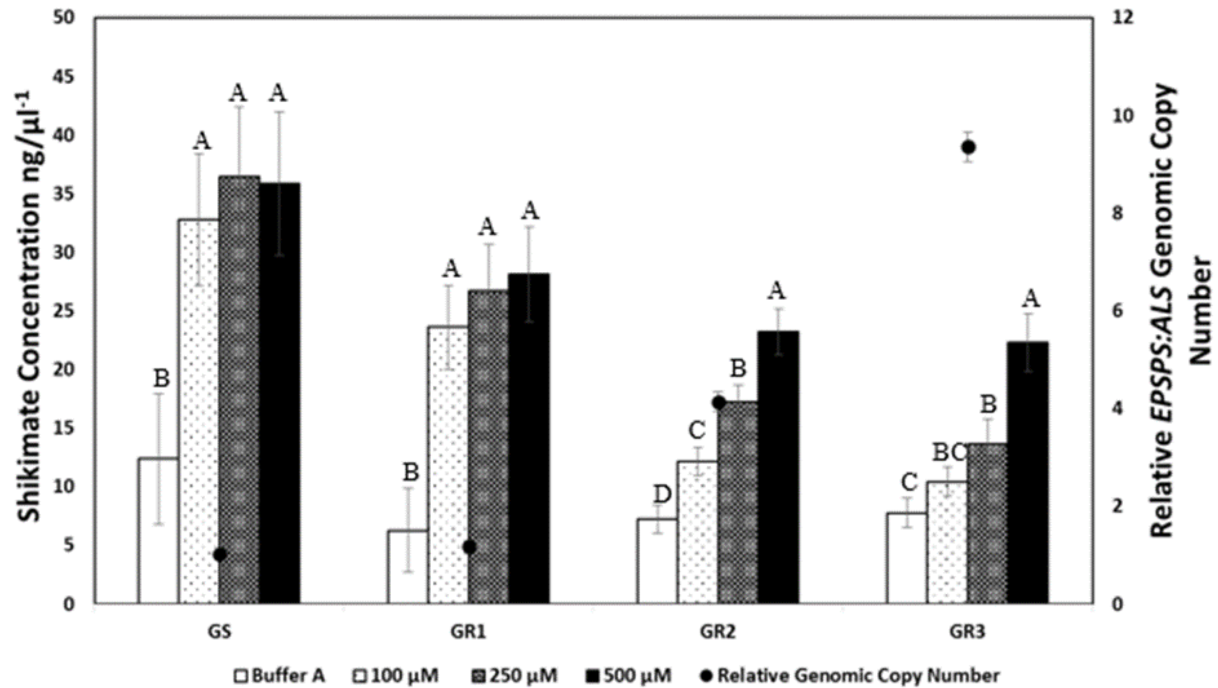

Figure 2.1: Combination bar chart and scatter plot showing the relationship between shikimate concentration (main Y-Axis), relative *EPSPS:ALS* genomic copy number (secondary Y-Axis), at several different concentrations of glyphosate for four resistance categories. GS is a group of four glyphosate-susceptible *A. rudis* plants with an average of 1.01 relative *EPSPS:ALS* genomic copies. GR1 is a group of seven *A. rudis* plants with low resistance to glyphosate with an average of 1.17 relative *EPSPS:ALS* genomic copies. GR2 is a group of sixteen moderately glyphosate-resistant *A. rudis* plants with an average of 4.13 relative *EPSPS:ALS* genomic copies. GR3 is a group of eight highly glyphosate-resistant *A. rudis* plants with an average of 9.35 relative *EPSPS:ALS* genomic copies. Different letters above the error bars denote significant difference from the other doses of that group.

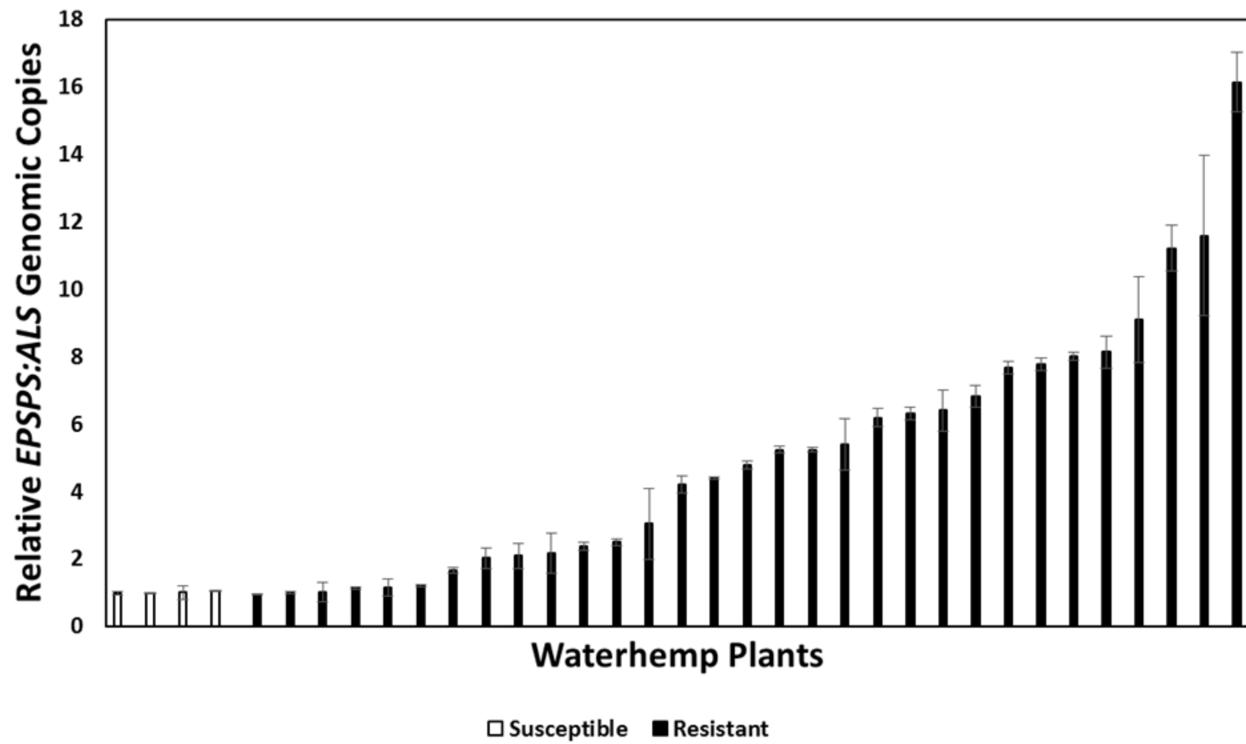

Figure 2.2: Bar graph of relative *EPSPS:ALS* genomic copy number (X-Axis) for *A. rudis* glyphosate-susceptible (white bars) and –resistant (black bars) plants.

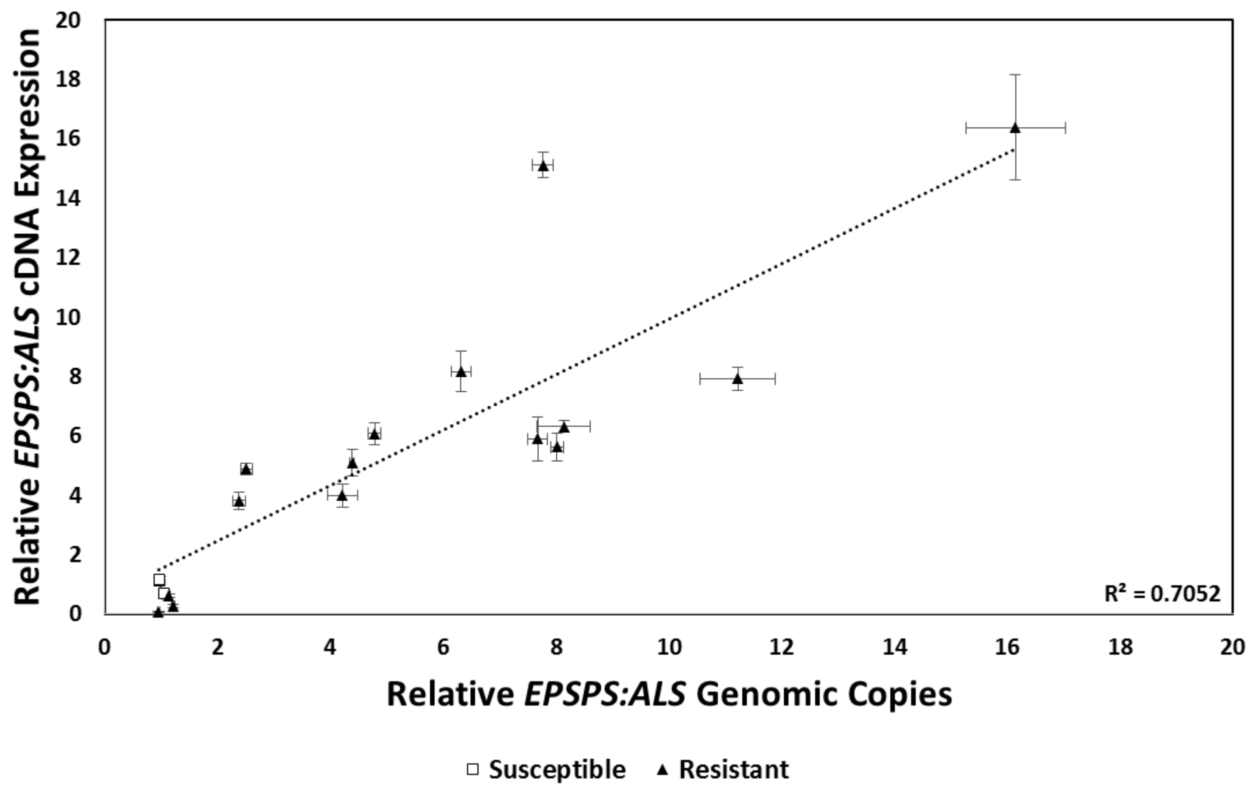

Figure 2.3: Scatter plot showing the relationship between relative *EPSPS:ALS* cDNA expression (Y-Axis) and relative *EPSPS:ALS* genomic copy number (X-Axis) for glyphosate-susceptible (white squares) and –resistant (black triangles) plants.

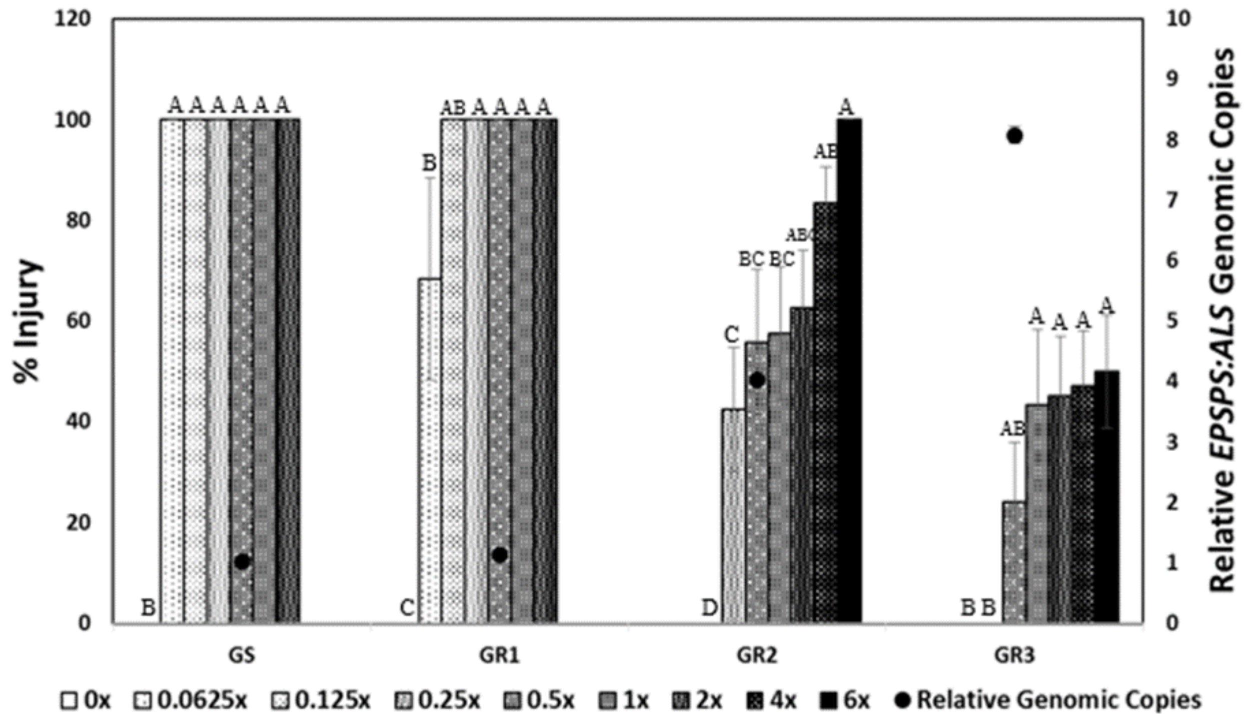

Figure 2.4: Combination bar graph and scatter plot showing the relationship percent visual injury (main Y-Axis), relative *EPSPS:ALS* genomic copy number (secondary Y-Axis), at several different doses of glyphosate for four glyphosate resistance categories. A 1x dose of glyphosate is 868 g ae ha^{-1}. GS is a group of three glyphosate-susceptible *A. rudis* plants with an average of 1.03 relative *EPSPS:ALS* genomic copies. GR1 is a group of four *A. rudis* plants with low glyphosate resistance, and has an average of 1.13 relative *EPSPS:ALS* genomic copies. GR2 is a group of six *A. rudis* plants moderately resistant to glyphosate with an average of 4.03 relative *EPSPS:ALS* genomic copies. GR3 is a group of five plants highly resistant to glyphosate with an average of 8.07 relative *EPSPS:*ALS genomic copies. Different letters above the error bars denote significant difference from the other treatments of that group.

```
WHSUSA  GCAGGAACAGCGACGCGCCCACCGACAGCTGCGGTT
WHSUSC  GCAGGAACAGCGACGCGCCCACCGACAGCTGCGGTT
WH3-B1  GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH3-1   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH3-2   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH3-3   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH3-4   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH7-A   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH7-1   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH7-2   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH7-3   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH7-4   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH7-6   GCAGGAACAGCGACGCGCCCATTGACAGCTGCGGTT
WH8-B   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-2   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-3   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-4   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-6   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-7   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-9   GCAGGAACAGCGATGCGCCCATTGACAGCTGCGGTT
WH8-11  GCAGGAACAGCGACGCGCCCACCGACAGCTGCGGTT
                          Pro106
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Figure 2.5: Segment of the *A. rudis EPSPS* gene showing the location of known mutation site for Proline 106, causing glyphosate resistance in species such as goosegrass and rigid rygegrass. The yellow-highlighted region indicates the codon for Proline, identical in both glyphosate-susceptible and –resistant plants. WHSUS (A, B) indicates susceptible plants from the control populations. WH 3 (B1, 1, 2, 3, 4) are plants from a population confirmed to have low glyphosate resistance. WH 7 (A, 1, 2, 3, 4, 6) are plants from a confirmed moderately glyphosate-resistant population. WH 8 (B, 2, 3, 4, 6, 7, 9, 11) are plants from population confirmed to be highly glyphosate-resistant.

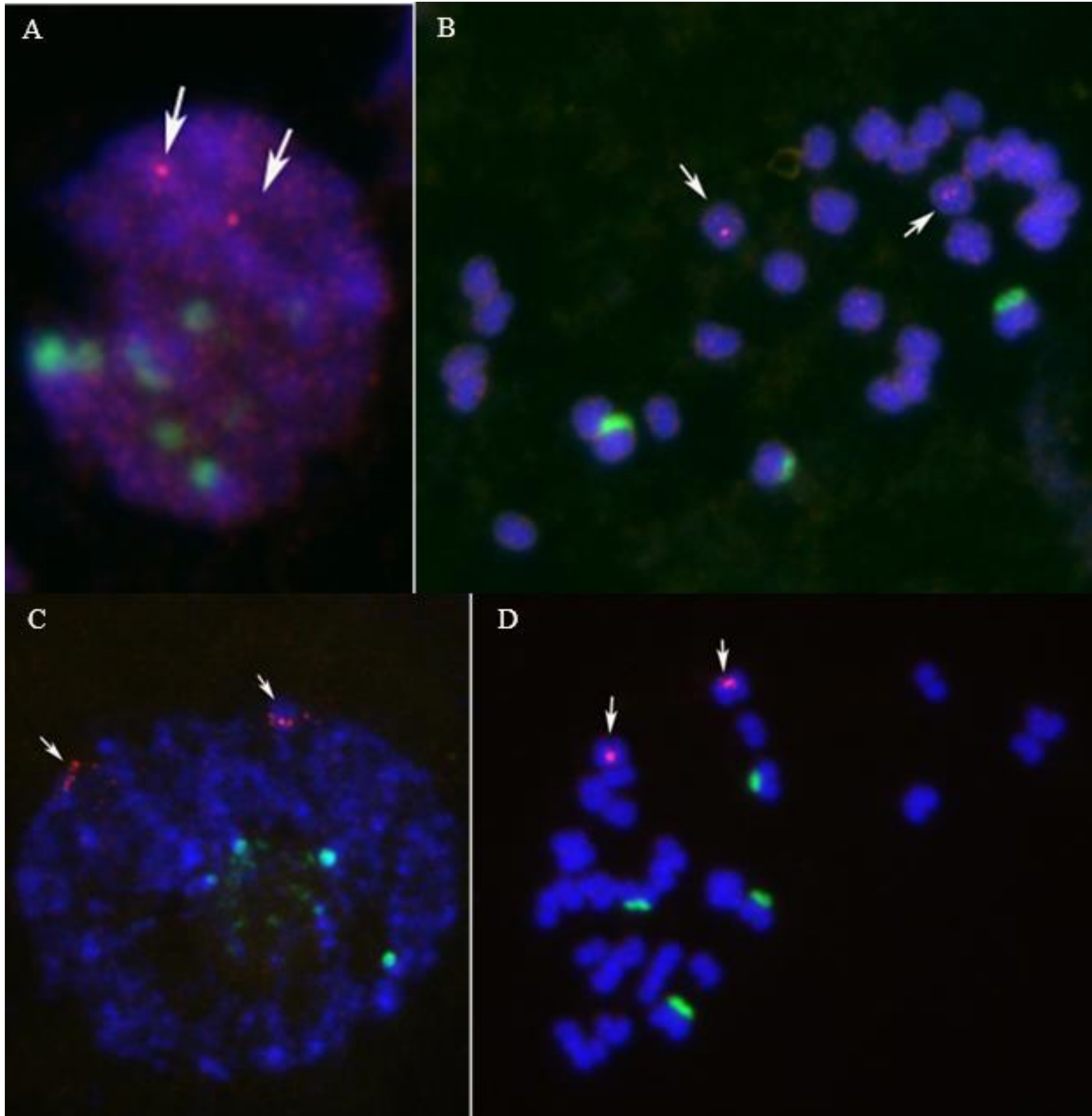

Figure 2.6: Florescence *in situ* hybridization images of stained somatic chromosomes of two *A. rudis* plants. Images A (prophase chromosomes) and B (metaphase chromosomes) are from a glyphosate-susceptible plant with one *EPSPS* copy relative to *ALS*. Images C (prophase chromosomes) and D (metaphase chromosomes) are from a glyphosate-resistant plant with nine *EPSPS* copies relative to *ALS*. Green signals are controls, signals in pink are *EPSPS*, indicated with white arrows.

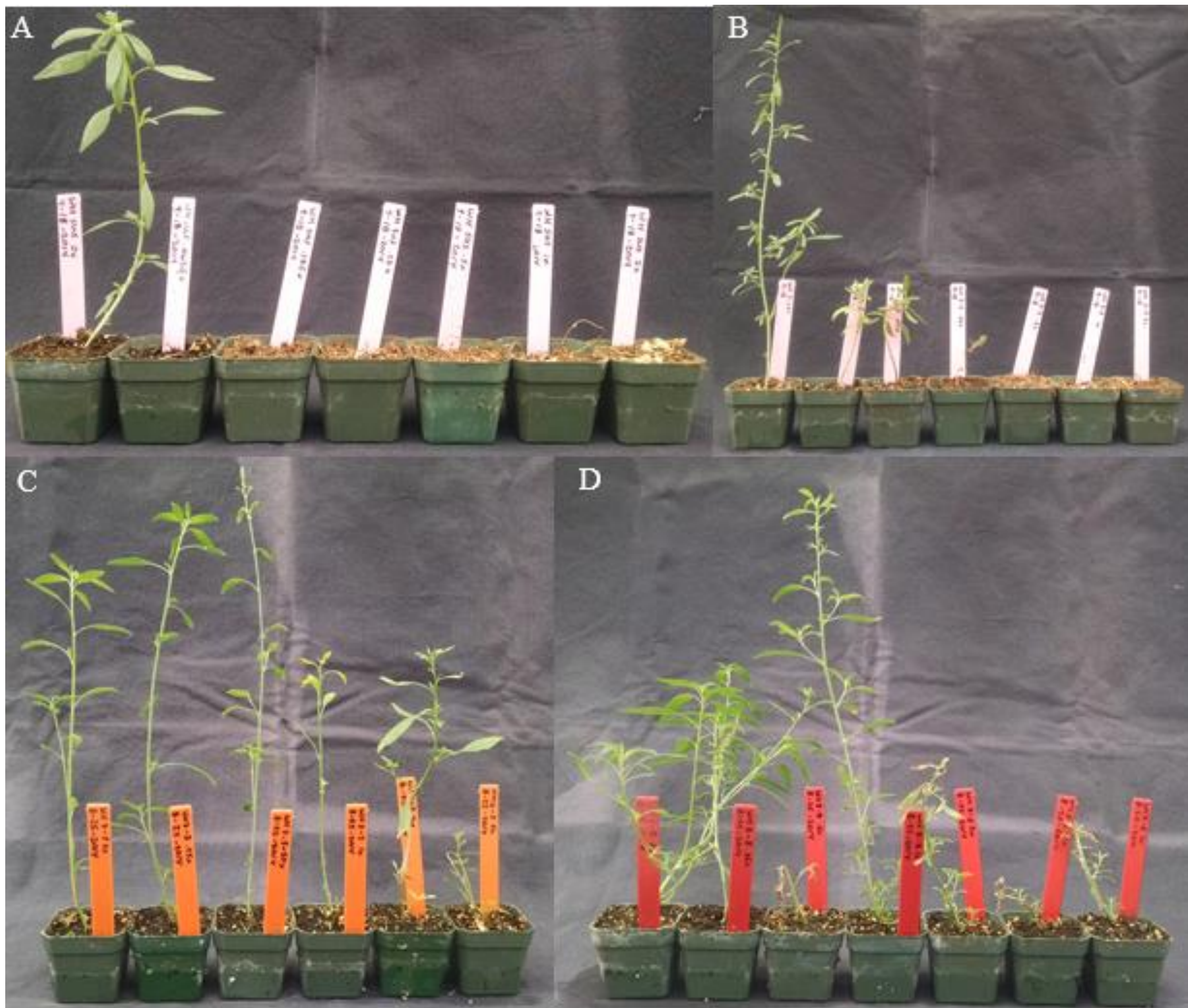

Figure 2.7: Images from whole-plant dose response studies on *A. rudis* clones. Image A) shows response to glyphosate treatment on clones of a single glyphosate-susceptible plant. Image B) shows response to glyphosate treatment on clones of a single plant with low glyphosate resistance. Image C) shows response to glyphosate treatment on clones of a single moderately glyphosate-resistant plant. Image D) shows response to glyphosate treatment on clones of a single highly glyphosate-resistant plant.

Tables

Table 2.1: Resistance classification and GPS coordinates of collections site for populations studied in Putman (2013).

Population	Resistance Category	GPS Coordinates
WH3	Low	N 39^{o} 50.498 W 96^{o} 51.921
WH7	Moderate	N 38^{o} 36.229 W 95^{o} 17.199
WH8	High	N 38^{o} 34.753 W 95^{o} 16.903

Table 2.2: List of glyphosate-susceptible and –resistant plants used in this research. Numbers in parenthesis are the standard error for that relative *EPSPS* gene copy number or gene expression value.

Plant	**Relative *EPSPS* Copy Number**	**Relative *EPSPS* Expression**
WHSUSC	0.979 (0.037)	1.13 (0.051)
WHSUSA	0.984 (0.017)	1.11 (0.136)
WHSUSB	1.04 (0.022)	0.676 (0.061)
WH3-4	0.953 (0.025)	0.080 (0.019)
WH3-3	1.13 (0.026)	0.627 (0.048)
WH3-1	1.20 (0.027)	0.259 (0.066)
WH7-4	2.37 (0.110)	3.81 (0.278)
WH7-6	2.50 (0.103)	4.88 (0.175)
WH7-2	4.21 (0.261)	4.00 (0.387)
WH8-11	4.38 (0.042)	5.10 (0.444)
WH7-3	4.78 (0.114)	6.08 (0.364)
WH7-1	6.31 (0.173)	8.17 (0.694)
WH8-8	7.67 (0.175)	5.90 (0.747)
WH8-6	7.78 (0.182)	15.12 (0.443)
WH8-2	8.01 (0.112)	5.62 (0.452)
WH8-7	8.13 (0.472)	6.32 (0.202)
WH8-9	11.22 (0.663)	7.92 (0.385)
WH8-4	16.14 (0.887)	16.37 (1.77)

Appendix A - Transfer of 2,4-D Tolerance from *Raphanus raphinistrum* into *Brassica napus*: Production of F_1 Hybrids Through Embryo Rescue

Abstract

Phenoxy herbicides [e.g. 2,4-dichlorophenoxy acetic acid (2,4-D) and 4-chloro-2-methylphenoxy acetic acid (MCPA)] are widely used for selective control of broadleaf weeds in agriculture. *Raphanus raphanistrum* (wild radish) a diploid member of Brassicaceae is a problem weed across the globe, specifically in Australia. As a result of extensive selection, 2,4-D-resistant- *R. raphanistrum* populations evolved in Western Australian wheat fields. However, the closely related *Brassica napus* (canola), a tetraploid brassica species, is a globally grown as an oilseed crop sensitive to phenoxy herbicides. The objective of this research was to generate hybrids between *B. napus* (2n:38) and 2,4-D-resistant *R. rphanistrum* (2n:18) via *in vitro* embryo rescue and to evaluate transfer of 2,4-D tolerance from *R. raphanistrum* into hybrids. Ten putative hybrid plants were produced *in vitro*; however, only six putative hybrids were established in greenhouse. Furthermore, DNA ploidy of the putative hybrids and parents was determined by flow cytometry. 2,4-D dose-response of putative hybrid clones indicate possible transfer of 2,4-D tolerance from wild radish into one of the putative hybrid plants. The results of this research has potential to develop 2,4-D-tolerant *B. napus* lines; and possibly other agriculturally important members of the Brassicaceae as well. Development of 2,4-D-tolerant Brassica crops will potentially be valuable for conservation crop production systems by providing herbicide rotation options to growers.

Introduction

Brassica crops such as oilseed rape (*B. napus*), Indian mustard (*B. juncea*), cabbage (*B. oleracea*) and turnip mustard (*B. rapa*), are grown widely across the globe. *B. napus* is extensively grown worldwide; nonetheless, weed competition can significantly reduce yield in this crop. Therefore, timely weed management is crucial for sustained yields in this crop. Glyphosate-resistant *B. napus* varieties are commercially available and are widely cultivated. However, increasing evolution of glyphosate-resistant weeds (Heap 2014) limits the long-term viability of this technology, and warrants need for development of new herbicide tolerance technology. Phenoxy herbicides such as 2,4-D (2,4-dichlorophenoxy acetic acid), and MCPA (4-chloro-2-methylphenoxy acetic acid) are cost-effective and do not have long soil residual activity. Recently, 2,4-D-tolerant corn and cotton cultivars were developed by introducing an enzyme aryloxyalkanoate dioxygenase from a soil bacterium *Ralstonia eutropha* that can cleave 2,4-D into non-herbicidal form (Wright *et al.* 2010). Development of 2,4-D-tolerant crop technology will be valuable for growers, as it facilitates greater herbicide rotation options to improve weed management.

Raphanus raphanistrum, (wild radish), is also a member of the Brassicaceae family, but is an economically important weed infesting diverse agro-ecosystems worldwide (Cheam and Code 1995). Many herbicides, including phenoxy herbicides such as 2,4-D (2,4-dichlorophenoxy acetic acid), and MCPA (4-chloro-2-methylphenoxy acetic acid), are extensively used to manage this weed. Phenoxy herbicides are cost-effective and do not have long soil residual activity. As a result of extensive and continuous use of phenoxy herbicides over 17 years in wheat (*Triticum aestivum*) and lupin (*Lupin angustifolius)* cultivation in Western Australia, some biotypes of *R. raphanistrum* have evolved resistance to these herbicides (Walsh *et al.* 2004). Phenoxy

resistance in *R. raphanistrum* is well characterized at physiological and genetic level. 2,4-D-resistant *R. raphanistrum* biotypes are approximately 10 times more resistant than the susceptible biotypes (Mithila *et al.* 2013; Walsh *et al.* 2004). Furthermore, phenoxy herbicide resistance in this weed species is controlled by a single-semi dominant trait (Mithila *et al.* 2013)

Brassicaceae members include diploid (e.g. *B. rapa*, *B. kaber,* and *R. raphanistrum*, etc.) as well as allotetraploids (e.g. *B. juncea*, and *B, napus*). The allotetraploids possess two of the three genomes; i.e., AA, BB, or CC. Previous research reported successful gene transfer among members of Brassicaceae (Prakash and Hinata 1980, Bing *et al.* 1995; Hu 2002; Snowdon *et al.* 2000; Tonguc and Griffiths 2004; Mithila and Hall 2013, 2014). Gene transfer may be complicated due to variation in chromosome numbers among the members of Brassicaceae, resulting in non-fertile hybrids (Mizushima 1950, 1980). However, *in vitro* techniques (e.g. ovule/embryo rescue) have been used for successful production of interspecific hybrids among *Brassica* members (Inomata 1988; Mathias 1991; Bing *et al.* 1995; Momotaz *et al.* 1998; Tonguc and Griffiths 2004; Mithila and Hall 2014). Hybrid plant formation among *Brassica* members has occurred via protoplast fusions as well (Hu *et al.* 2002), although natural crossings have been reported in rare instances (Myers 2006). This research was initiated based on the hypothesis that introgression of the 2,4-D tolerance trait from *R. raphanistrum* into commercially valuable *B. napus* should be possible using *in vitro* embryo rescue and regeneration technique. Thus, the overall objectives of this research were to: i) to generate hybrids between *B. napus* (2n:38) and 2,4-D-resistant *R. rphanistrum* (2n:18) via *in vitro* embryo culture and regeneration; ii) determine the DNA ploidy to identify true hybrid plants; and iii) confirm the transfer of 2,4-D tolerance from *R. raphanistrum* into hybrids.

Materials and Methods

Phenoxy-resistant *R. raphanistrum* and -susceptible *B.napus* [e.g. winter (Kiowa) and spring (Flint) canola varieties] were grown from seed in a greenhouse. The seeds were sown in 15 cm plastic pots containing commercial potting mixture (Miracle Gro, Marysville, OH) and were grown under 15/9 h day/night photoperiod, supplemented with 250 µmol $m^{-2}s^{-1}$ illumination provided with sodium vapor lamps. Each pot contained one plant. When plants were flowering, crosses were performed between *B. napus* and phenoxy-resistant *R. raphanistrum* following the procedure described by Jugulam *et al.* (2005). Embryo rescue was required for hybrid plant production *in vitro*. Silique (immature seed pod), ovule culture, and putative hybrid plant regeneration was established according to the procedure described by Mithila and Hall (2014). Plantlets produced *in vitro* were transferred to soil and were grown in a greenhouse. Upon putative hybrid plant establishment, clonal propagation was achieved by single nodal cutting.

DNA ploidy of putative hybrids and parental plants was assessed by flow cytometry using a BD FACS Calibur flow cytometer (BD Biosciences, San José, USA). Fresh leaf tissue was chopped in LB01 buffer (Doležel *et al.* 1989) containing 50 µg/mL PI and 50 µg/mL RNAse, fluorescence area (585/42nm) was measured, and 2C DNA content was determined relative to an internal standard of Zea mays 'CE-777' (5.43 pg/2C; Lysak & Doležel, 1998). Because the DNA content of the standard exceeded that of 2C *R. raphanistrum* by more than 3 times, *R. raphanistrum* 4C peaks were used to measure genome size in order to maintain linearity. A minimum of 1,000 nuclei per peak was obtained and all CV's<4.2%. DNA ploidy of putative hybrids was determined by comparison of 2C DNA content to known *R. raphanistrum* diploids and *B. napus* tetraploids.

To determine if tolerance to 2,4-D from *R. raphanistrum* was successfully transferred into putative hybrids, 2,4-D dose-response experiment was conducted using putative hybrid clones. Parental plants (*B. napus* and *R. raphanistrum*) as well as putative hybrid clones were grown in a greenhouse (previously described). The putative hybrid clones and parental seedlings (at 4 leaf stage) were treated with 2,4-D [125, 250 and 500 g acid equivalent per hectare (ae/ha)] using a bench-type sprayer as described by Mithila *et al.* (2013). Following 2,4-D treatment, plants were returned to the greenhouse. The putative hybrid plants were classified as phenoxy resistant or susceptible by comparing their injury responses with those of *R. raphanistrum* or *B. napus* plants.

Results and Discussion

Ovule/embryos rescued from cultured immature siliques facilitated putative hybrid plant production and establishment (Fig. A.1 A). A total of 10 putative hybrid plants were produced *in vitro*, with a higher number of putative hybrids produced from crosses between the *B. napus* winter canola variety (Kiowa) and *R. raphanistrum* compared to the spring canola (Flint) (8 vs 2 hybrids, respectively; Table A.1). We determined DNA ploidy of six of these hybrids (as the other four did not establish in soil) along with the parents (*B. napus* and *R. raphanistrum*). We estimated the DNA content of 4X *B. napus* as 2.40 pg/2c and 2X *R. raphanistrum* as 1.06 pg/2c, and calculated an expected 3X value of 1.73 pg/2c based on these results (Table A.2 and Fig A.2). Only three hybrid plants were found to be DNA triploids, with DNA contents between 1.71 and 1.74 pg/2c (Table A.2 and Fig A.2). The other three putative hybrids were found to be DNA tetraploids, with estimated DNA content close to *B. napus* (2.41-2.44 pg/2c; Table A.2). These DNA tetraploids may have been true hybrids resulting from the union of an unreduced *R. raphanistrum* gamete and a reduced *B. napus* gamete, or they may have been derived from

somatic tissue of the immature ovule rather than the fertile embryo. The 3X hybrids exhibited several morphological traits of both the parents (leaf shape, stem, and plant height; Fig. A.1 B-D), and one of the 3X hybrids (#1) also survived up to 250 g ae/ha of 2,4-D application (Fig. A.3). After treatment with 2,4-D, the hybrid #1 exhibited little or no epinasty (downward curling of plant parts; a typical symptom of auxinic herbicides) and this response was similar to 2,4-D-resistant *R. raphanistrum* plants. The 2,4-D-resistant 3X hybrid plant continued to grow normally and produced flowers, but there was no pollen production, nor was the stigma fertile. Therefore, these plants could not be used in introgression.

Here, we report for the first time successful production of hybrid plants between *B. napus* and *R. raphanistrum* and possible transfer of 2,4-D tolerance into one of the hybrids generated via embryo rescue. This is the first step towards development of 2,4-D tolerant *B. napus* varieties following this approach. Future research will help increase the frequency of embryo regeneration and hybrid plant production. Also, techniques such as chromosome doubling with colchicine can be utilized to improve fertility of 2,4-D-resistant hybrids. The outcome of this research is encouraging for future development of 2,4-D-tolerant *B. napus* cultivars, which may allow farmers to use this herbicide both as pre-emergence as well as post-emergence. Furthermore, such technology will also provide herbicide rotation options to growers and can facilitate effective weed control, less tillage, and possibly minimize evolution of herbicide resistant weeds.

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Michael Stamm, KSU Canola Breeding for supplying greenhouse space and Kiowa winter canola seed and North Central Regional Plant Introduction Station for supplying Flint spring canola seed.

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Figures

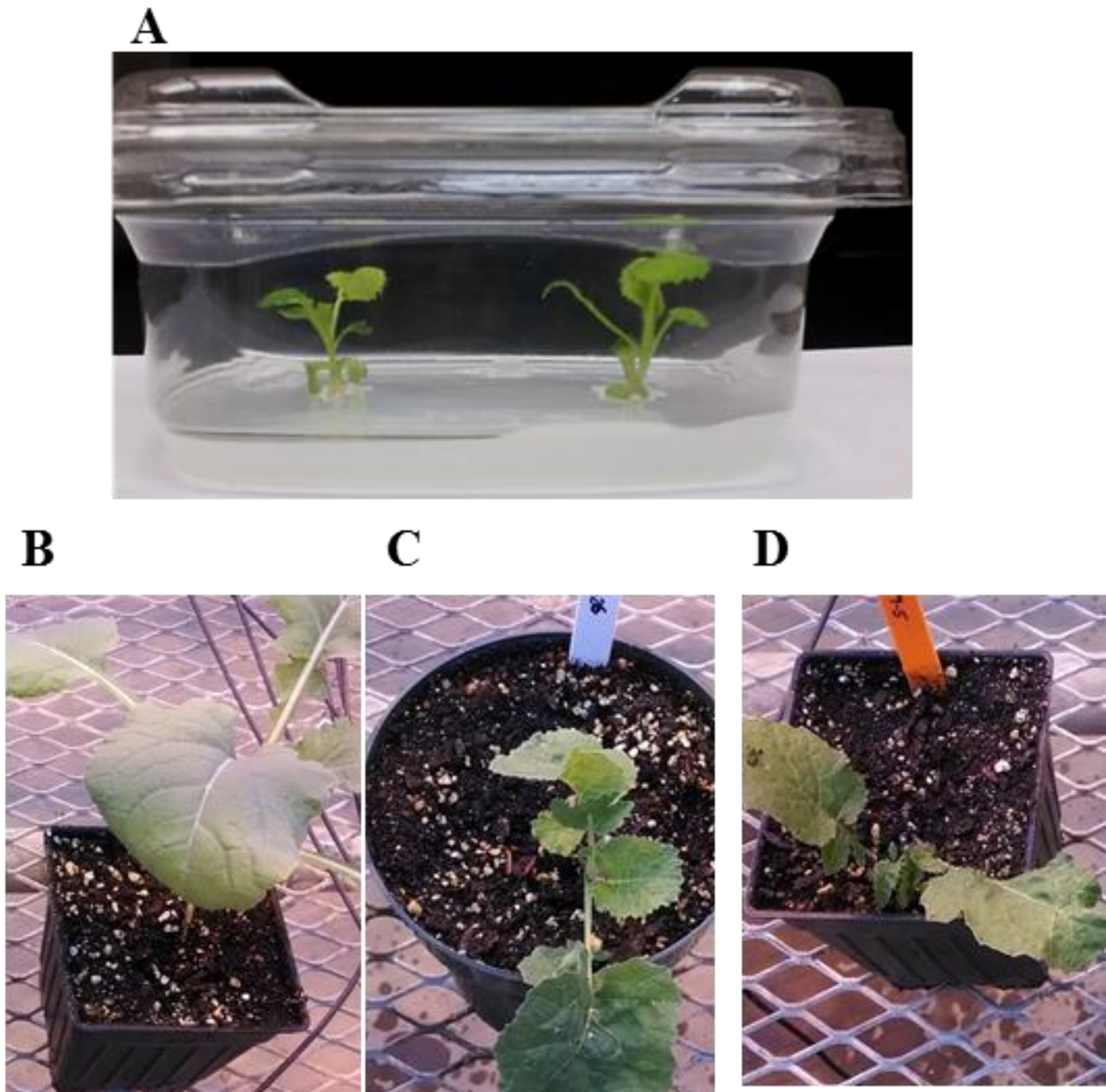

Figure A.1: Production of hybrids between *Brassica napus* and *Raphanus raphanistrum*. A illustrate hybrids produced via embryo rescue. B–D represent *B. napus*, F_1 hybrid (#1) and *R. raphanistrum*, respectively. Note the hybrid #1 exhibiting intermediate characteristics of parents.

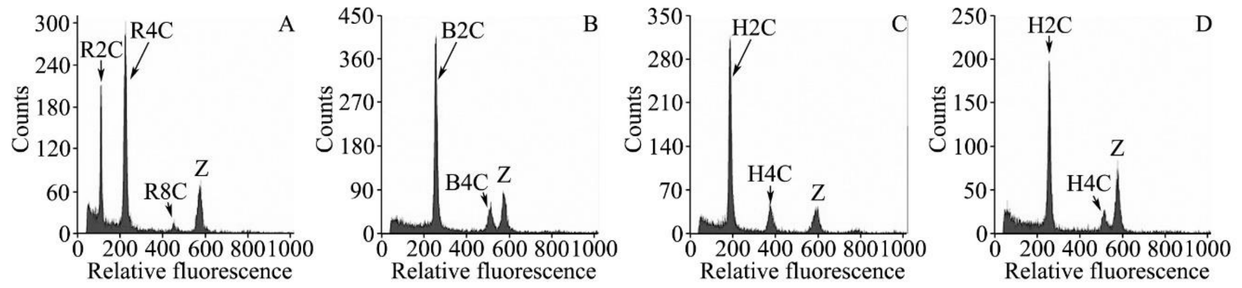

Figure A.2: Figure 2: Flow cytometry relative fluorescence (fluorescence area) histograms for nuclei extracted from (A) diploid *R. raphanistrum*, (B) tetraploid *Brassica napus*, (C) a triploid hybrid with 2C DNA content intermediate to (A) and (B), and (D) a putative hybrid with 2C DNA content matching *B. napus* (B). Z is the 2C peak for *Zea mays*, included as an internal DNA content standard; R, B and H indicate *R. raphanistrum*, *B. napus* and putative hybrid nuclei peaks; 2C, 4C and 8C peaks are indicated for R, B and H.

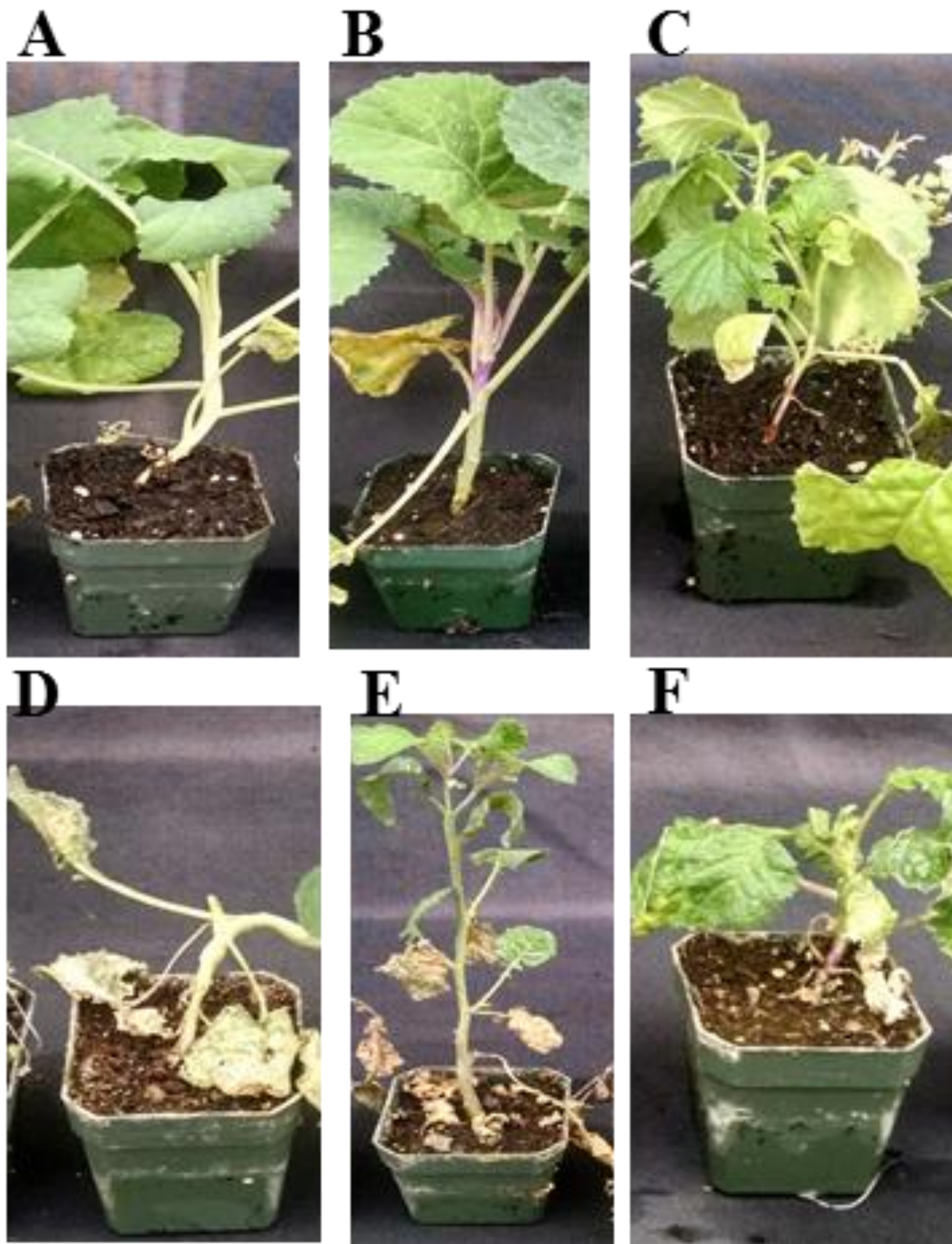

Figure A.3: Plant response of untreated or treated with 250 g ae/ha of 2,4-D. A-C represent untreated *B. napus*, triploid hybrid #1, *R. raphanistrum,* respectively. D-F show the response of *B. napus*, triploid hybrid #1, *R. raphanistrum* 3 weeks after treatment with 2,4-D (250 g ae/ha).

Tables

Table A.1: Hybrid production between *B. napus* and *R. raphanistrum*: Frequency of embryo regeneration and hybrid plant establishment via *in vitro* embryo rescue (based on protocol from Mithila and Hall, 2013)

Cross combination	**# of buds pollinated**	**# of siliques cultured**	**# of embryos excised**	**# of embryos germinated**	**# of putative hybrids established**
B. napus (winter canola variety) × *R. raphanistrum*	400-450	50-60	25-30	8	5
B. napus (spring canola variety) × *R. raphanistrum*	600-625	140-150	60-100	2	1

Table A.2: 2C DNA content and DNA ploidy of parents and hybrids. [a] expected 2C DNA content of a triploid hybrid calculated as the average of the two *B. napus* and two *R. raphanistrum* means.

Plants tested	DNA content (pg/2c) mean and 95%CI	DNA Ploidy level
Parents:		
B. napus (plant 1)	2.37 (2.37, 2.37)	4X
B. napus (plant 2)	2.43 (2.22, 2.64)	4X
R. raphanistrum (plant 1)	1.06 (1.04, 1.08)	2X
R. raphanistrum (plant 2)	1.07 (1.03, 1.11)	2X
Predicted 3X hybrid[a]	1.73	3X
Putative Hybrids:		
B. napus (winter) × R. raphanistrum #1	1.73 (1.72, 1.73)	3X
B. napus (winter) × R. raphanistrum #4	1.71 (1.64, 1.77)	3X
B. napus (spring) × R. raphanistrum #1	1.74 (1.73, 1.74)	3X
B. napus (winter) × R. raphanistrum #2	2.41 (2.38, 2.44)	4X
B. napus (winter) × R. raphanistrum #3	2.44 (2.15, 2.72)	4X
B. napus (winter) × R. raphanistrum #5	2.42 (2.35, 2.50)	4X